Institut für Veterinärphysiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. M. Gassmann
Arbeitsgruppe: Prof. Dr. T. A. Lutz

Diet-derived protein and amino acids attenuate the responsiveness of the area postrema to the anorectic hormone amylin in male rats

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Karoline Forster

Tierärztin
aus Wien, Österreich

genehmigt auf Antrag von
PD Dr. Thomas Riediger, Referent
Prof. Dr. Heinz Augsburger, Korreferent

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Contents

1 Summary

The pancreatic β-cell hormone amylin decreases food intake via activation of brainstem neurons in the area postrema (AP). Diet-derived protein is thought to attenuate amylin responsiveness. Here we investigated the influence of isocaloric diets with differing protein contents on the amylin–dependent AP activation (c-Fos expression) and food intake suppression. Further, we investigated whether circulating amino acids affect these amylin actions. Finally, we characterized the possible interaction between amylin and glucagon, which is secreted in particular after a protein intake.

1. The amylin-induced c-Fos expression is attenuated in rats fed an isocaloric diet with 18% protein content compared to rats fed with the 1% protein diet. Peripherally injected amino acids also attenuated the amylin-mediated AP activation.
2. Amylin's anorectic action was stronger in rats fed the 1% protein diet compared to its action in rats fed an isocaloric diet with higher protein content (8 and 18% protein).
3. Glucagon did not induce a c-Fos response in the AP and it had no effect on the amylin-dependent activation of AP neurons.

This study extends the concept that diet-derived protein attenuates neuronal amylin responsiveness and amylin's anorectic action, independently of caloric intake. Glucagon does not seem to be a mediator in this mechanism. Because the use of amylin analogs is a promising approach for the treatment of obesity the current findings might be therapeutically relevant.

2 Zusammenfassung

Das Pankreashormon Amylin hemmt die Nahrungsaufnahme über die Aktivierung von Neuronen in der Area postrema im Hirnstamm. Nahrungsproteine scheinen die Amylinsensitivität abzuschwächen. Wir untersuchten den Einfluss von isokalorischen Diäten mit unterschiedlichem Proteingehalt auf die Amylin - vermittelte AP-Aktivierung (c-Fos Expression) und die anorektische Wirkung von Amylin. Ebenfalls wurde untersucht, ob zirkulierende Aminosäuren diese Amylinwirkungen beeinflussen und ob es eine mögliche Interaktion zwischen Amylin und Glukagon gibt, welches v.a. nach Proteinaufnahme sezerniert wird.

1. Die Amylin induzierte c-Fos Expression war bei der 18% Protein Diät schwächer als nach Gabe der 1% Protein Diät. Peripher injizierte Aminosäuren schwächten die Amylin vermittelte c-Fos Expression ebenfalls ab.
2. Amylin's anorektische Wirkung war stärker bei der 1% als bei der 8% oder der 18% Proteindiät.
3. Glukagon induzierte keine c-Fos Expression in der AP und hatte auch keinen Einfluss auf die von Amylin abhängige Aktivierung der AP Neurone.

Die vorliegende Studie sichert die Hypothese, dass Protein die neuronale Amylinresponsivität und die anorektische Wirkung von Amylin abschwächt. Dieser Effekt hängt nicht von der Kalorienaufnahme ab. Glukagon scheint keinen Einfluss auf diesen Mechanismus zu haben. Da Amylin-Analoga einen vielversprechenden Ansatz zur Bekämpfung der Fettleibigkeit darstellen, sind die vorliegenden Ergebnisse eventuell auch therapeutisch relevant.

3 Introduction

The long term balance of energy intake and energy expenditure is a fundamental requirement for a normal body function. Food intake provides macronutrients that are used as metabolic fuels and to maintain normal cellular structure and function (Woods et al., 2000). Various control signals that reflect the body's energy status act at different target sites in the body to maintain energy homeostasis. Generally, the multitude of signals is categorized into satiation and adiposity signals. Endogenous factors that physiologically control the size of an ongoing meal are considered satiation signals (Woods et al., 2004). Cholecystokinin (CCK) is the best investigated hormonal satiation signal in this regard. CCK is released from the duodenum in response to food intake. Further, the pancreas produces the two anorectic peptide hormones amylin and glucagon that both reduce meal size when administered systemically (Geary and Smith, 1982; Weatherford and Ritter, 1988; Morley and Flood 1991; Lutz et al., 1994; Lutz et al. 1995). There are two major ways how these peptides signal to the central nervous system (CNS). These signals involved in the control of energy balance may reach the brain via the neural pathway or by direct humoral action on the brain. CCK and glucagon, for example, act via afferent vagus nerve fibers, which transmit the excitatory input to the hindbrain. Amylin and some metabolic factors act via the humoral pathway.

The two best investigated hormones that are considered adiposity signals are leptin, which is secreted from white adipose cells and insulin which is secreted from pancreatic β - cells (Woods et al., 2004). Adiposity signals fulfill the following criteria. They circulate at levels proportional to body fat and enter the CNS in proportion to their plasma level. Administration of either peptide directly into the brain reduces food intake, whereas deficiency of either hormone leads to an opposite effect (Schwartz et al., 2000). Leptin and insulin are believed to act primarily on neurons located in the hypothalamic arcuate nucleus where they interact with neurons that synthesize the proopiomelanocortin (POMC) derived anorectic neuropetide alpha-MSH or the orexigenic neuropeptide Y (NPY), repectively.

3.1 The area postrema / nucleus tractus solitarii region and its projections

The area postrema (AP), which is located on the dorsal surface of the medulla oblongata at the caudal end of the brain stem, belongs to the circumventricular organs (CVO) that are specialized areas of the brain with fenestrated capillaries. The CVO are subdivided into sensory and neurosecretory structures. The first group comprises the AP, the subfornical organ (SFO) and the organum vasculosum laminae terminalis. The eminentia mediana, the pineal organ and the posterior pituitary, or neurohypophysis, are classified as neurosecretory CVO (Fry and Ferguson, 2007).

All CVO lack a functional blood-brain barrier (BBB). The BBB typically isolates the CNS from the rest of the body and protects the CNS from changes in the peripheral electrolyte concentrations, from

toxins and also from pathogens. The BBB also prevents brain intrinsic neuropeptides, transmitters and growth factors from diffusing out of the CNS into the general circulation (Fry and Ferguson, 2007).
One of the first recognized roles of the AP was its function as the chemoreceptor trigger zone, which is involved in the induction of nausea and emesis as protective mechanisms against intoxications (Harding et al., 1985; Miller and Leslie, 1994). More importantly for the work presented here, the AP is an important target area for circulating hormones. In particular, it is the primary target site for amylin, which directly activates AP neurons (Rowland et al., 1997; Riediger et al., 2004) (see below).
The nucleus of the solitary tract (NTS), which is located in close proximity to the AP, is reciprocally connected to the AP via monosynaptic projections (Ferguson, 1991). Therefore, it is a site of convergence for satiation signals acting via the vagus nerve (e.g. CCK) and via the AP (e.g. amylin). Further connections from the NTS project to the lateral parabrachial nucleus (lPB), the central nucleus of the amygdala (Ce) and the lateral subdivisions of the bed nucleus of the stria terminalis (BSTL) (Riediger et al., 2004). These brain structures (AP, NTS, lPB, Ce, BSTL) are part of the central gustatory/enteroceptive system and are mutually interconnected and linked to the hypothalamus, the main integrative centre for the control of energy balance (Saper et al., 2002).

3.2 Amylin

The molecular structure of amylin was discovered in 1987 (Cooper et al., 1987; Westermark et al., 1987). Amylin is a 37 amino acid peptide hormone which is co-secreted with insulin from pancreatic β-cells at a molar ratio of approximately 100:1 (insulin : amylin) in response to food intake (Butler et al., 1990; Kahn et al., 1990; Ludvik et al., 1997).
Amylin is a member of the calcitonin peptide family, together with calcitonin, α and β calcitonin - gene related peptide (CGRP) and adrenomodullin (AM) (Wimalawansa, 1997). Amylin binds to the calcitonin core receptor (CTR) with ligand specificity being conferred by receptor activity modifying proteins (RAMP's) that heterodimerize with the CTR receptor (Chen et al., 1997; Christopoulos et al., 1999).
Functional amylin receptors are named AMY $_{1(a),}$ AMY $_{2(a),}$ AMY $_{3(a);}$ (a) stands for the heterodimer of the type a calcitonin receptor (CTa), the numbers 1, 2, 3 indicate the association with RAMP 1, RAMP 2 or RAMP 3 (Poyner et al., 2002).
Autoradiographic studies by (Sexton et al., 1994) demonstrated that amylin binding occurs with high density in the sensory CVO's and the ventromedial hypothalamic nucleus. Becskei (Becskei et al., 2004) investigated the distribution of the CTR gene in adult rat brains by immunohistochemical methods. She found an abundant expression of CTR in many areas of the brain, with high densities in the AP/NTS region.
One of amylin's best-investigated functions is its inhibitory effect on food intake which is mediated by the AP (Morley and Flood, 1991; Lutz et al., 1994). At physiological plasma levels, amylin also

inhibits glucagon secretion (Fehmann et al., 1990; Young, 2005a) and reduces the rate of gastric emptying (Young, 2005 b). Similar to amylin's anorectic action, these effects are also thought to be mediated by the AP.

Amylin activates AP neurons after peripheral administration (Rowland et al., 1997; Michel et al., 2007); most of these studies made use of the immunohistological detection of the immediate early gene product c-Fos as a marker of neuronal activation (Hoffman et al., 1993; Hughes and Dragunow, 1995). Interestingly, endogenously released amylin also seems to activate the AP because a refeeding induced c-Fos expression in 24h fasted rats has been shown to be attenuated in rats pre-treated with a specific amylin receptor antagonist (Riediger et al., 2004). In line with this observation, intravenous infusion of an amylin receptor antagonist leads to an increase in food intake suggesting that endogenous amylin physiologically inhibits feeding (Mollet et al., 2004). At the cellular level it has been demonstrated that amylin leads to an excitation of AP neurons, which seems to be mediated by an intracellular formation of the second messenger cGMP (Riediger et al., 2001).

3.3 Therapeutic approaches using amylin agonists

Obesity is a major health problem in humans and animals worldwide and is considered as an epidemic by the World Health Organization (WHO, 2009). The prevalence of obesity is rising and promotes the development of severe secondary health problems like cardiovascular disease, type II diabetes, sleep apnea, musculoskeletal disorders and some cancers, as well as many psychosocial disorders (Aronne et al., 2009). Based on the finding that amylin and insulin are normally cosecreted from pancreatic β cells, amylin replacement is used as an adjunct therapy with insulin in type I and type II diabetes therapy. Due to its ability to lower the rate of gastric emptying and to inhibit postprandial glucagon secretion (Young, 2005a), amylin slows down the flux of carbohydrates into the circulation (Young and Denaro, 1998) and hence contributes to an improved control of postprandial glycemia compared to insulin alone. The amylin agonist pramlintide is approved in the USA as a therapeutic drug for the treatment of human type II diabetes.

Apart from surgical approaches (e.g. Roux-en-Y gastric bypass surgery or gastric banding (Benotti et al., 1989; Peterli et al., 2009)), the development of therapeutic strategies for the treatment of obesity has not been very successful in the past. Several pharmacological approaches were either not effective or were associated with major undesired side effects so that several drugs have recently been withdrawn from the market. However, new promising hormonal treatment strategies emerged recently that are based on combined administration of amylin and leptin agonists. Preclinical rodent studies and clinical trials with humans have shown that a combination therapy of amylin and leptin is more potent than the weight-lowering effect of amylin or leptin alone (Roth et al., 2007; Roth et al., 2008; Ravussin et al., 2009). These findings not only indicate that amylinergic drugs are important

therapeutic targets for the treatment of obesity and associated metabolic disorders, but they also imply that the clinical efficacy of amylin is modulated by other factors.

3.4 Aim and hypothesis

The potency of some anorectic hormones depends on the macronutrient composition of the diet. For example, centrally administered glucagon-like peptide 1 (GLP-1) reduced food intake when given concurrently with a meal or a preload of carbohydrate but not when a protein preload was given (Choi and Anderson, 2001). Similarly, the effect of CCK seems to be nutrient-dependent because CCK inhibited and the CCK_A receptor antagonist L-364,718 enhanced carbohydrate intake, but not protein intake when offered as an intra-oral amino acid infusion (Mamoun et al., 1997). Most studies investigating the anorectic effect of amylin after acute treatment used standard rodent chow (Rushing et al., 2001) or a medium fat diet (Lutz et al., 1998a; Lutz et al., 1998b) without directly comparing amylin's feeding inhibitory action under different diet conditions. It has been demonstrated, however, that under chronic amylin treatment rats not only decreased their energy intake, but that they also shifted their intake preference from a high-fat diet to low-fat dietary components (Mack et al., 2007).

In a recent study conducted by our group (Michel et al., 2007) amylin's potency to reduce food intake and to induce c-Fos expression in the AP/NTS region was affected by the macronutrient content of the diet. A low dose of peripheral amylin (5μg/kg) induced a strong c-Fos expression in the AP/NTS in 24 hrs fasted rats, but not in rats that were fed ad libitum. The same dose also induced a strong c-Fos response in rats that received a nutrient deficient diet called non caloric mash (NCM) for 24 hrs before injection. As determined by selective nutrient supplementation to NCM, protein seemed to attenuate both the amylin-induced activation of the AP/NTS region and the inhibitory effect on feeding. The same was not observed with glucose or fat added to NCM. Interestingly, the protein dependent attenuation of amylin's anorectic effect did not occur in chow-fed rats although the intake of protein was similar in these animals when compared to the protein intake of rats fed the protein supplemented non-caloric diet. These findings put forward the hypothesis that the inhibitory effect of protein on amylin's anorectic effect and possibly on other AP-mediated functions might be modulated by the presence of other macronutrients (carbohydrates and fat) in the diet. In other words, when isocaloric diets are offered, the ability of amylin to reduce food intake and to activate the AP might increase with decreasing protein content of the diet.

Therefore it was one aim of the present study to investigate the influence of proteins and of an amino acid mixture on amylin induced c-Fos expression and amylin's anorectic action in more detail. We tested the influence of three isocaloric diets which differed only in their protein content (18%, 8%, 1%), on the amylin induced activation of the AP and on amylin's effect to reduce food intake. Furthermore we sought to determine whether an acute rise in plasma amino acids following an intraperitoneal injection of an amino acid mixture is sufficient to attenuate amylins neuronal

responsiveness. A negative outcome of the latter study would suggest that a direct effect of circulating diet-derived amino acids is not important for the protein-dependent attenuation of amylin-responsiveness.

Because glucagon plasma levels are particularly elevated during the course of protein meals in rats and humans (Geary et al., 1981), and because amino acids (especially arginine, alanine and glutamine) are potent stimulators of glucagon secretion (Pipeleers et al., 1985; Young and Denaro, 1998; Dumonteil et al., 2000) we assumed that glucagon might play a role in the modulation of amylin induced c-Fos expression in the AP/NTS region.

Summary of specific questions:

Do isocaloric diets that differ in their protein content influence amylin's anorectic effect and the amylin - induced c-Fos expression in the AP/NTS?

Do peripherally administered amino acids influence amylin's anorectic effect and the amylin - induced activation (c-Fos expression) in the AP?

Does glucagon modulate the amylin - induced c-Fos expression in the AP/NTS region?

4 Animals, Material and Methods

4.1 Animals and housing

Male Wistar rats (Elevage Janvier, Le - Genest - St - Isle, France) were used in all experiments; their mean body weight was about 300 g at the start of the experiments. The rats were adapted to the housing conditions and to handling (taking them out of the cage, weighing, performing injections) for at least seven to ten days before the experiments started.

The animals had ad libitum access to tap water and to standard laboratory rodent chow (3430; Provimi Kliba, Gossau, Switzerland **Table 1**), except during periods when the animals received specific test diets or during food deprivation as described below. All rats were individually housed in hanging, stainless steel wire cages (50 x 25 x 18 cm) under controlled conditions of illumination (12:12 h light – dark cycle), humidity and temperature (21 ± 1°C). The Veterinary Office of the Kanton Zurich, Switzerland, approved all animal procedures and experiments.

4.2 Diets

In addition to standard chow, different test diets were used.

We used three isocaloric diets (Provimi Kliba AG; Kaiseraugst, Switzerland), which differed in their protein content (18%, 8%, 1%; **Table 1**) and a diet called non-caloric mash (NCM) (Michel et al., 2007) for the feeding and c-Fos studies. NCM is composed of 2.5 parts by weight α-cellulose, 1.0 part mineral oil, 10.0 parts deionised water solution containing 0.1% sodium saccharin, and 0.2% vanilla extract (Altromin, Lage, Germany). The NCM diet was freshly prepared before each experiment. Prior to each experiment, the animals were adapted to the new diets in the habituation period. During this period, the diets were offered two times for 24 hrs, together with standard chow; during one additional day immediately before the test phase, the diet was offered alone.

Table 1 *Compositions of standard laboratory chow and the three isocaloric diets of different protein content used in the c-Fos and feeding studies (NfE = nitrogen free extract). Distributed by Provimi Kliba AG, Kaiseraugst, Switzerland.*

		chow	18% protein	8% protein	1% protein
in % (w/w)					
dry matter		**88**	**90**	**90**	**90**
	protein	18.5	18	8	0.7
	fat	4.5	4.5	4.5	4.5
	ash	6.3	3.0	3.1	3.1
	starch	35	38	48	58
	NFE	54.2	61	70.90	78.15
	simple sugars (sucrose, dextrose)	19.2	23	22.9	20.15
	fiber	4.5	3.5	3.5	3.5
energy ME (MJ/kg)		13.2	15.4	15.2	15.1

4.3 Technical procedures

4.3.1 Immunohistochemistry (c-Fos studies)

To investigate c-Fos expression in the AP/NTS region, weight-matched animals were either food-deprived or fed with the 1% and 18% protein diets respectively. The animals were maintained under these conditions for 24 hours starting from the beginning of the dark phase one day before injections. At the onset of the following dark phase, the rats received a s.c. injection (1ml/kg) of rat amylin (5µg/kg; Bachem, Bubendorf, Switzerland) or NaCl (NaCl 0.9% B. Braun, B. Braun Medical AG, Sempach, Switzerland) for control. Amylin was dissolved in 0.9% NaCl.

Two hours after the respective treatments, the animals were deeply anaesthetized with pentobarbital sodium (100 mg/kg i.p.; Pentobarbital sodium, 50 mg/ml; Kantonsapotheke Zürich, Switzerland); the absence of reflexes (tail and toe reflex) was tested. Then, i.e. after reaching surgical tolerance, the abdomen was opened and the diaphragm was cut. After cutting the costal arches laterally on both sides, the thorax was opened. The left ventricle of the heart was penetrated with a cannula (Terumo 18G x 1/2, Leuven, Belgium) and ice-cold phosphate buffer solution (PB 0.1 M, pH 7.2) was infused for 1.5 min; the right atrium was opened before the heart was incised with a pair of scissors. The perfusion of PB was followed by ice-cold 4% paraformaldehyde solution (4% PB 0.1 M) for in situ fixation of the tissue for 2 min.

The brains were removed and post fixed for additional two hours in a 4% paraformaldehyde solution (4% PB 0.1 M) at 4 °C. After incubation for 48 hours in 20 % sucrose in PB for cryoprotection, the brains were transsected between the cerebrum and the cerebellum. Both parts of the brain were put on parafilm strips partly covered with tissue freezing medium and snap frozen with CO_2 for approximately 1 min.

Two series of 20 µm coronal sections of the medulla oblongata at the level of the AP/NTS (Paxinos et al., 2007) were cut in a cryostat (Leica CM 3050 S, Nussloch, Germany), thaw mounted on adhesive glass slides (Super Frost Plus; Menzel Braunschweig, Germany) and stored at -20°C.

After air drying for one hour at room temperature the slides were rehydrated in phosphate buffered saline (pH 7.4) containing 0.1% Triton X – 100 (PBST) for half an hour. The sections were incubated either in normal goat serum or in normal rabbit serum (1.5% in 0.3% PBST) for two hours to prevent unspecific binding.
Two different primary antibodies were used for the detection of c-Fos expression. Initially, immunohistological detection of c-Fos expression was performed using a goat polyclonal anti-c-Fos antibody IgG (Santa Cruz Biotechnology, Heidelberg, Germany) in 0.3% PBST (1:10.000) for 48 hrs at 4 °C. However, due to a high non-specific background staining with this antibody, a second primary antibody (rabbit polyclonal anti-c-Fos (Ab-5) (4-17) (Calbiochem, Darmstadt, Germany) in 0.3% PBST (1:5.000) was employed in later experiments.
After washing in 0.1% PBST (5 x 10 min), the brain sections were incubated with the secondary antibody (in 1:1.000 biotinylated rabbit anti goat antibody, Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, California USA or in 1:400, biotinylated goat anti rabbit, Vector Laboratories) for 90 minutes at room temperature. After 1h incubation in avidin-biotin complex (Vectastain Elite ABC Kit), c-Fos was visualized by incubation in biaminobenzidine solution (0.04% buffered saline with 0.02% H_2O_2, 0.08% NiCl, and 0.01% CoCl; Sigma Aldrich) for five to seven minutes. The slides were rinsed in PBST, dehydrated in graded alcohol, immersed in xylol, and cover slipped with Entellan (Merck, Darmstadt, Germany). All incubations were done on Plexiglas plates (Max Planck Institut, Bad Nauheim, Germany) in closed and wet metal boxes.

C-Fos stainings were evaluated using a microscope equipped with a digital camera (Axioskop; Carl Zeiss, Feldbach, Switzerland). The experimenter was blinded to the experimental groups. C-Fos positive cells were counted in every fourth section of the AP/NTS region, resulting in approximately six sections per animal (AP/NTS – bregma -14.30 mm to - 13.56 mm). The cell counts were averaged for each animal. Group mean values ± SEM were calculated from the averaged cell counts of each animal for each treatment group (n = number of animals).

4.3.2 Blood sampling

Blood samples were taken from each rat by cardiac puncture just before perfusion or from the lingual vein under isoflurane anaesthesia. Immediately after blood collection, the concentration of glucose was measured using a glucose-oxidase based glucometer (Glucometer Elite; Bayer, Zürich). The blood

was transferred into serum tubes (Microvette 500 Z, Sarstedt, Germany) and centrifuged at 2800 rpm for ten minutes. The serum was then removed and frozen at -20 °C.

When hormone blood levels were measured with a LINCOplex Kit (Rat endocrine kit for measuring: insulin, glucagon, amylin, leptin; rat gut kit for measuring: peptide tyrosine tyrosine, pancreatic polypeptide; Millipore Corporation, Billerica, MA, USA); a protease inhibitor cocktail (P 2714, Sigma - Aldrich) was immediately added after blood sampling (10 µl of the inhibitor / 1 ml blood).

4.3.3 Immunoassay for plasma hormone levels (LINCOplex KIT)

The rat endocrine (Cat. No.: RENDO - 85K) and the rat gut (Cat. No.: RGT – 88K) LINCOplex assays (Millipore corporation, Billerica, MA, USA) were used for the analysis of serum samples. The LINCOplex assay is a multianalyte detection system and was used according to the manufacturer's instructions. Briefly, the assay is based on conventional sandwich assay technology and permits simultaneous detection of multiple hormones from a single sample. The antibody specific for the hormone of interest is covalently coupled to Luminex micro spheres uniquely labelled with fluorescent dye.

The microspheres were incubated overnight (17 hrs) at 2-8°C with standards, controls and the serum samples (10 µl of the sample for the endocrine kit and 25µl of the sample for the gut hormone kit) in a 96-well microtiter filter plate with agitation on a plate shaker.

After incubation, the plate was washed three times with an assay wash buffer (200 µl/well) to remove excess reagents between each washing step and prior to the addition of the detection antibody (50 µl/well). After sixty minutes of incubation on a plate shaker at room temperature, 50 µl streptavidin – phycoerythrin were added to each well containing 50 µl of detection antibody cocktail, and incubated for thirty minutes.

After a final washing step (washing and removing fluids by vacuum filtration 3 times), the beads were resuspended in buffer (100 µl sheath fluid) and the plate was analyzed using the Luminex 100 analyzer to determine the concentration of the hormones of interest.

4.3.4 Amino acid measurements

The measurements of amino acid concentrations were performed by the Functional Genomics Centre (FGCZ) at the University of Zurich in Switzerland. 80µl of each serum sample were used and 80µl of a 10% sulfasalicyclic acid solution (SSA) was added for precipitation. 20µl of the supernatant was taken and 60µl borate buffer were added to the vial and reconstituted with 20µl MassTrak Amino Acid Analysis Solution derivatization reagent. 1µl of this vial was injected into the Waters Acquity Ultra Performance Liquid Chromatography (UPLC) machine equipped with an UV detector (Waters Corporation, Milford, MA, USA) and the samples were analyzed. Analyses were carried out according to the manufacturer's instructions.

4.4 Experimental design

4.4.1 Effect of isocaloric diets with different protein contents on the anorectic effect of amylin and on the amylin-induced c-Fos expression in the AP/NTS

Blood sampling for measurements of amino acid baseline levels and plasma hormone levels at the onset of the dark phase

First the animals were adapted to the new diets, which were offered twice during two dark phases in a habituation period of seven to ten days. Then the animals received chow for another 48 hrs. After this period, the animals received chow and the test diet for 24 hrs and were then fed the special diets only for 48 hrs beginning at dark onset; blood sampling was conducted at dark onset the other day to measure the baseline amino acid levels (tongue blood).
For the measurement of plasma hormone levels after an amylin injection, rats were treated as described above. At dark onset, they received an injection of 5µg/kg amylin s.c. and 20 min later blood was collected from the tongue vein.

Effect of the three isocaloric diets that differ in their protein content on amylin's anorectic effect

The anorectic effect of amylin was tested in a crossover design. The animals were adapted for seven to ten days to the experimental conditions. During this time the animals received the isocaloric diets that differed in protein content on two days for 24 hrs beginning always at the beginning of dark onset. After this habituation period, the animals were allocated to two weight-matched groups and a period of another 48 hrs of chow feeding followed. Then the animals received another 24 hrs chow and the test diet. At the day of testing, all animals had been exposed to the test diet for 24 hrs and diet groups were then subdivided into one amylin-treated (5µg/kg s.c.) group and one control group receiving saline (n = 7 for all groups). Injections were at dark onset. The cumulative food intake was measured 30 min, one hour and two hours after injection with a precision of 0.1g and correction for spillage. The animals were kept on the diet until 24 hrs after the injection and then switched back to chow. After a two-day period of chow feeding the rats were fed according to the same feeding regimen; but at the next trial at onset of the dark phase, the treatment groups were exchanged and tested using a crossover design. After another period of two days of chow feeding, this paradigm was repeated to test the effects of the next diet (order diets: trial 1 1% protein diet, trial 2 18% protein diet, trial 3 8% protein diet (**Figure 1**)).

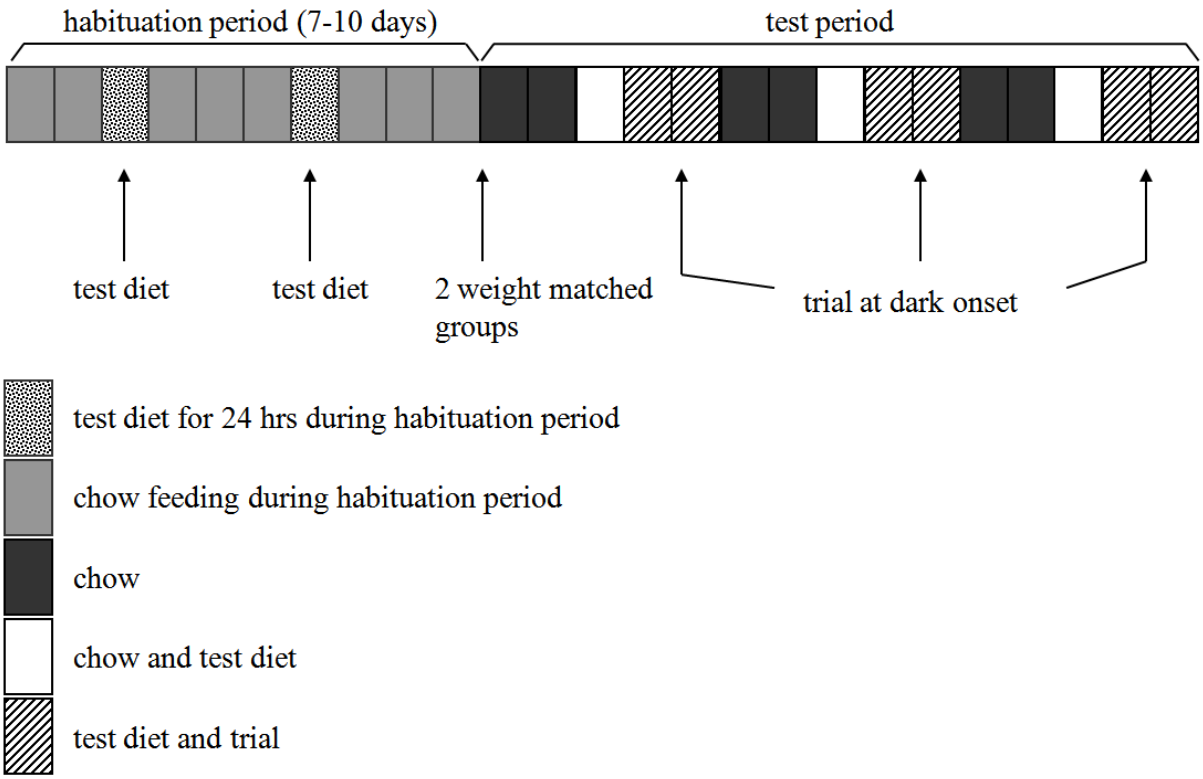


Figure 1 *Example of how a feeding study was conducted*

Interaction of an 18% and 1% protein diet with amylin and the effect on c-Fos expression in the AP

In this experiment we tested whether rats showed a difference in the amylin-induced c-Fos expression in the AP, depending on the protein content of the diet (**Table 1**). The animals received either the 1% or the 18 % protein diet for 48 hrs according to the regime used in the feeding study. Each of these diet groups was then subdivided into one amylin-treated (5µg/kg s.c.) group and one control group receiving saline (n = 7 for all groups) at dark onset. Rats were allowed to continue feeding for 2 hours until sacrifice (perfusion).

4.4.2 Effect of peripherally administered amino acids on the anorectic effect of amylin and on the amylin-induced c-Fos expression in the AP

Blood amino acid levels after injection of two different doses of amino acid

We first performed a pilot experiment to investigate the time course of blood amino acid levels after the injection the of two different doses of the amino acid mixture. We used a commercial amino acid solution Aminoven 15% (Fresenius Kabi Schweiz AG) which contains 16 different amino acids. This

solution is used in human medicine for parenteral nutrition. It contains eight essential, two semi-essential and six non-essential amino acids (for further information see **Table 2**).
The animals were food-deprived for 24 hrs and divided into three groups, including a control group (n = 4) that did not receive Aminoven, and two groups injected with 1g/kg or 2g/kg Aminoven (n = 8 for both groups).
Aminoven 15% solution was injected intraperitoneally with sterile syringes (Omnifix, Braun Melsungen AG, Melsungen, Germany) and sterile needles (Terumo, 26G, Leuven, Belgium).

Blood was sampled just before (0 min) and 20 min and 60 min after injection. At each time point, different animals were used. The animals were anaesthetized with isoflurane and blood was taken by cardiac puncture. Amino acid, blood glucose and hormone levels (insulin, amylin, leptin, glucagon, PP, PYY) were determined.

Table 2 *Content of amino acids in Aminoven 15% (150g total amino acids/litre solution) distributed by Fresenius Kabi Schweiz AG*

Aminoven 15%

	g/mol	g/l	mol/l
l-isoleucine	131.17	5.2	0.039643211
l-leucine	131.18	8.9	0.067845708
l-lysine	146.19	11.1	0.075928586
l-methionine	149.21	3.8	0.025467462
l-phenylalanine	165.19	5.5	0.033294994
l-threonine	119.12	8.6	0.072196105
l-tryptophane	204	2	0.007834381
l-valine	117.15	5.5	0.046948357
arginine	174.2	20	0.114810563
l-histidine	155.16	7.3	0.047048208
alanine	89.09	25	0.280615108
glycine	75.07	18.5	0.246436659
l-proline	115.13	17	0.147659168
l-serine	105.09	9.6	0.091350271
l-tyrosine	181.19	0.4	0.002207627
taurine	125.14	2	0.0159821
total	2183.508	150	1.315268509

Effect of Aminoven on amylin's anorectic effect

To test the effect of prior Aminoven injection (20 min before dark onset) on amylin's anorectic effect, we conducted a feeding study with 24 animals divided into four weight-matched groups. The groups had been adapted to their diets for seven to ten days. During this time the animals received the 1% protein diet twice. 48 hrs before the feeding trials, the animals received again the 1% protein diet; then, they received the respective Aminoven, amylin or control treatments. Cumulative food intake

was measured 30 min, 1 hr and 2 hrs after injections with a precision of 0.1g and correction for spillage. After a 2-3 day period of chow feeding, the rats were fed the same diet again for 48 hrs; at the onset of the dark phase, the treatment groups were exchanged and tested in a crossover design.

Effect of Aminoven on amylin-induced c-Fos expression in the AP

To test the effect of an Aminoven injection on amylin-induced c-Fos expression in the AP, four weight-matched groups including a saline control group (n=5), an amylin-treated group (n=7), an Aminoven control group (n=8) and a group that was treated with Aminoven and amylin (n=7) were used. The Aminoven dose was chosen according to the results of the blood amino acid level measurements after injection of two different amino acids. The 1g/kg dose was chosen because it induced a rise of amino acid levels without affecting blood glucose levels.
The animals were food-deprived for 24 hrs and injected with Aminoven (1g/kg i.p.) or saline 20 min before dark onset. At dark onset, amylin (5 µg/kg s.c.) or saline were injected. Two hours after these treatments, the animals were deeply anaesthetized and transcardially perfused. Blood was sampled and the glucose concentration was measured as described.

4.4.3 Effect of glucagon on the amylin-induced c-Fos expression in the AP

In this experiment, we investigated if an acute injection of glucagon (Gluca Gen® Novo Nordisk, 1mg Küsnacht, Zürich; Switzerland Charge No. XW60072) influences the amylin induced c-Fos expression in the AP. In accordance with previous studies (Geary and Smith, 1982; Langhans et al., 1986; Lutz et al., 1996), we chose glucagon doses of 100µg/kg i.p. or 500µg/kg i.p. for the injections. The animals were food deprived for 24 hrs and allocated into six groups (saline / saline; saline / amylin; glucagon 100µg/kg / saline; glucagon 100µg/kg / amylin; glucagon 500µg/kg / saline; glucagon 500µg/kg / amylin). The rats received glucagon or NaCl 0.9% 10 min before dark onset. At dark onset, rats received either 5µg/kg of amylin or NaCl 0.9% injection as control; two hours later, the animals were perfused.

4.5 Statistical Analysis

All results are presented as mean values ± SEM. Differences in food intake were analyzed using a paired Student's *t*-test to compare the differences between amylin-treated and their control groups at each time point. Differences in amino acid or hormone levels between the treatment groups were analyzed using an unpaired *t*-test. For all other studies, the results were compared separately for each experiment using one way ANOVA or one way ANOVA on ranks for data that were not normally distributed, followed by the Student-Newman-Keuls post hoc test. In the immunohistological studies, the mean value of the cell counts/section of an individual animal was used for statistical analyses. In

all cases $p < 0.05$ was considered significant. Data were statistically analyzed using the software Prism Version 5.

5 Results

5.1 Effect of isocaloric diets with different protein contents on the anorectic effect of amylin and on the amylin-induced c-Fos expression in the AP/NTS

Blood amino acid levels for different test diets

The first experiment tested the effect of 48 hrs intake of diets with differing protein content on the concentration of amino acids in the blood. At the onset of the dark phase, blood samples were taken from the tongue vein. We observed a significant difference in the total blood concentration of amino acids among the three isocaloric diets that differed in their protein content (mean values: 1% protein diet 3102 ± 257.3 µmol/l (n=4); 8% protein diet 3969 ± 83.2 µmol/l (n=6); 18% protein diet 5562 ± 534.2 µmol/l (n=5); standard laboratory chow (approximately 18.5 % protein) 5306 ± 343.7 µmol/l (n=4)). Hence, chow fed rats had similar blood amino acid concentrations than rats fed the 18% protein diet. For illustration see **Figure 2**. Importantly, the blood glucose concentration did not differ significantly among rats receiving the three isocaloric diets different in protein content (**Figure 3**).

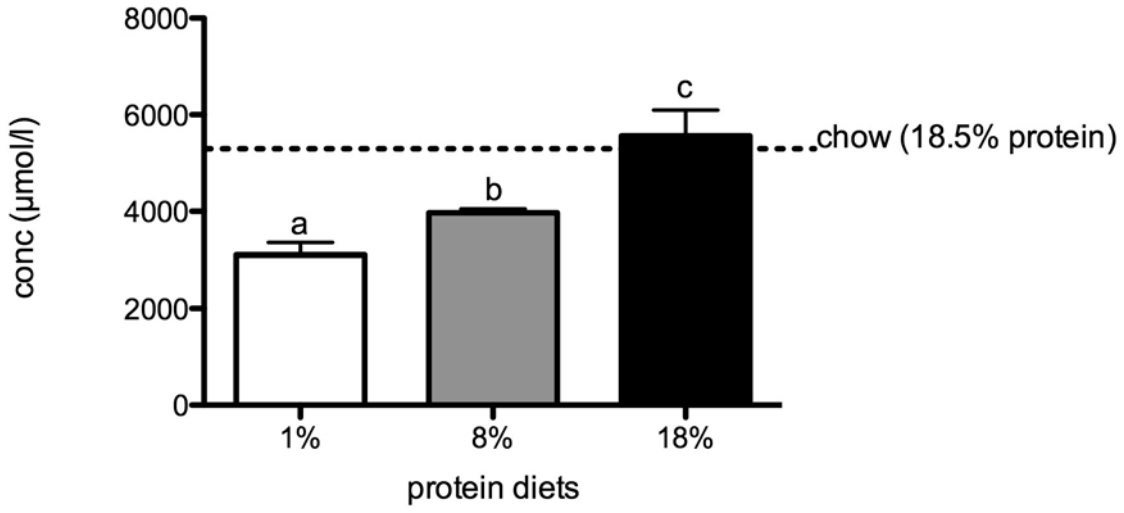


Figure 2 *Blood level of total amino acids measured in animals receiving the different test diets for 48 hrs; chow (dotted line) has a similar protein content as the 18% protein diet and resulted in similar blood amino acid levels. Data are expressed as means ± SEM. Different letters indicate significant differences ($p < 0.05$; one way ANOVA) (n: 1% = 4 ; 8% = 6; 18% = 5; standard laboratory chow =4) .*

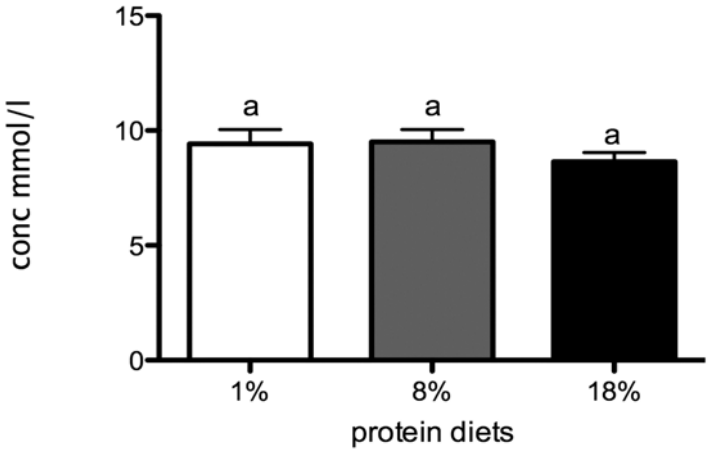


Figure 3 *Average blood glucose concentration measured in animals receiving the respective diets (see Figure 2) for 48 hrs. Data are expressed as means ± SEM. Bars with different letters are significantly different ($p < 0.05$; one way ANOVA) (n: 1% = 4 ; 8% = 6; 18% = 5)*

Plasma amylin levels after amylin injection in rats fed the different test diets

The increase in plasma amylin levels produced by the amylin injection in rats fed with the different protein diets for 48 hrs was significant in all amylin treated animals (1% protein diet: saline injected 6.9 ± 3.4 pM (n=3), amylin injected 161.5 ± 36.9 pM (n=6); 8% protein diet: saline injected 10.8 ± 3.5 pM (n=6), amylin injected 121.0 ± 23.9 pM (n=6); 18% protein diet: saline injected 8.3 ± 1.2 (n=4), amylin injected 133.7± 30.8 (n=6)). There were no significant differences among groups in saline or amylin treated rats, respectively. See **Figure 4**.

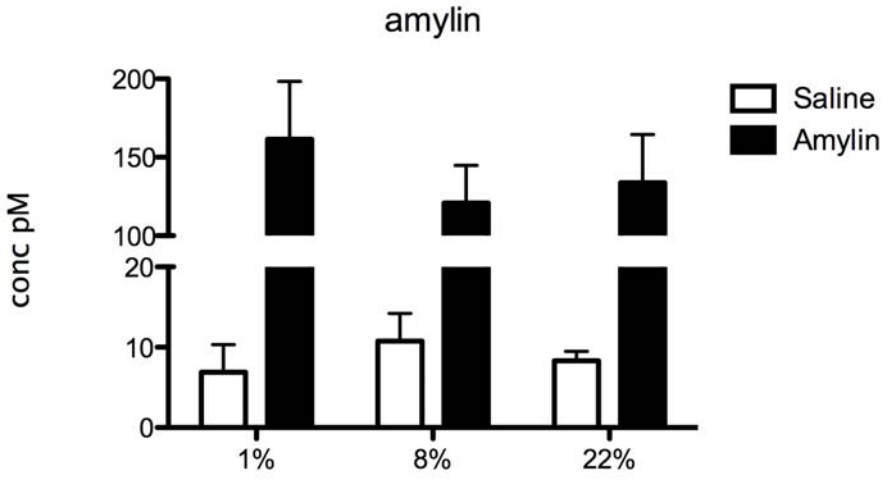


Figure 4 *Blood amylin levels after an amylin injection of 5ug/kg s.c. in rats fed the different test diets for 48 hours. Amylin was measured 20 min after injection.*

Effect of the three isocaloric diets different in their protein content on amylin's anorectic effect

Baseline food intake of rats eating the three isocaloric diets that differed in protein content was measured over 48 hrs. Data showed that rats ate the same amount of food of the 1% protein diet and of the 18% protein diet, but that rats receiving the 8% protein diet ate significantly less than rats on the other diets (mean food intake values: 1% protein diet 9.9 ± 0.6 g; 8% protein diet 7.6 ± 0.1 g; 18% protein diet 9.2 ± 0.3 g). See **Figure 5**.

Amylin significantly reduced food intake across all groups , i.e. in rats fed the 1%, the 8% or the 18% diet. The amylin-induced reduction in food intake was significant after 30 min and 60 min under all three conditions (**Figure 6**). After 120 min, amylin only reduced intake in the rats fed the 1% protein diet, but not when the 8% or the 18% protein diets were offered.

When compared to the pertinent control groups, amylin caused a stronger relative suppression of food intake in rats fed the 1% protein diet than in animals receiving the 8% or 18% protein diets (**Figure 7**). This effect was significant at 30 min when comparing the 1% vs. the 8% protein diet group, and after 60 min vs. both the 8% and the 18% diets.

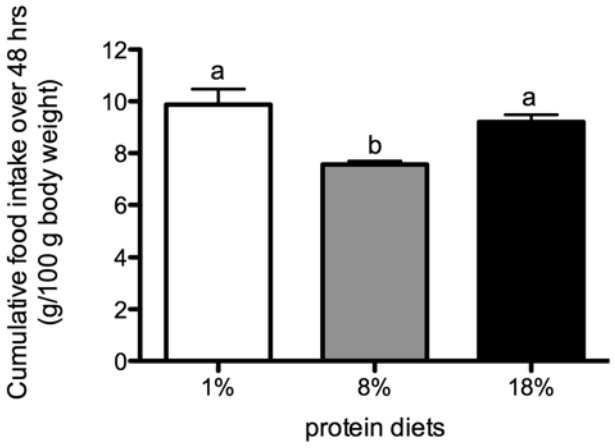


Figure 5 *Average food intake of the three isocaloric diets that differed in protein content over 48 hrs before each food intake trial. Data are expressed as means ± SEM. Bars with different letters are significantly different ($p < 0.05$; one way ANOVA) (n = 12 for 1%, 8% and 18%).*

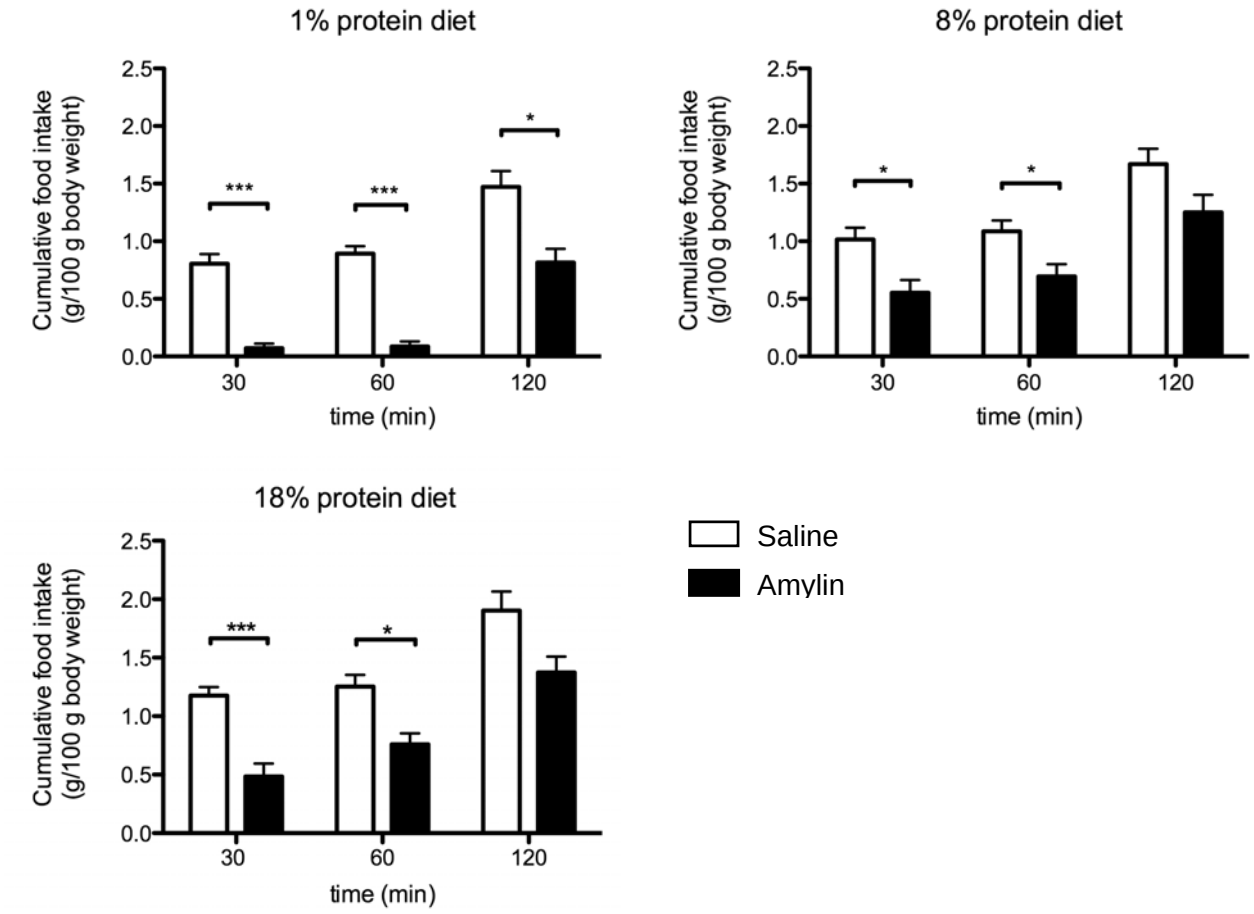


Figure 6 *Effect of amylin (5µg/kg s.c.) on food intake in rats kept on different test diets (1%, 8%, 18%) for 48 hrs prior to injection. Bars represent group means ± SEM. (n = 12: 1% and 18%; n = 10: 8 %) *p<0.05, and ***p<0.001 significantly different from respective control (saline) group at individual time points (paired Student t test).*

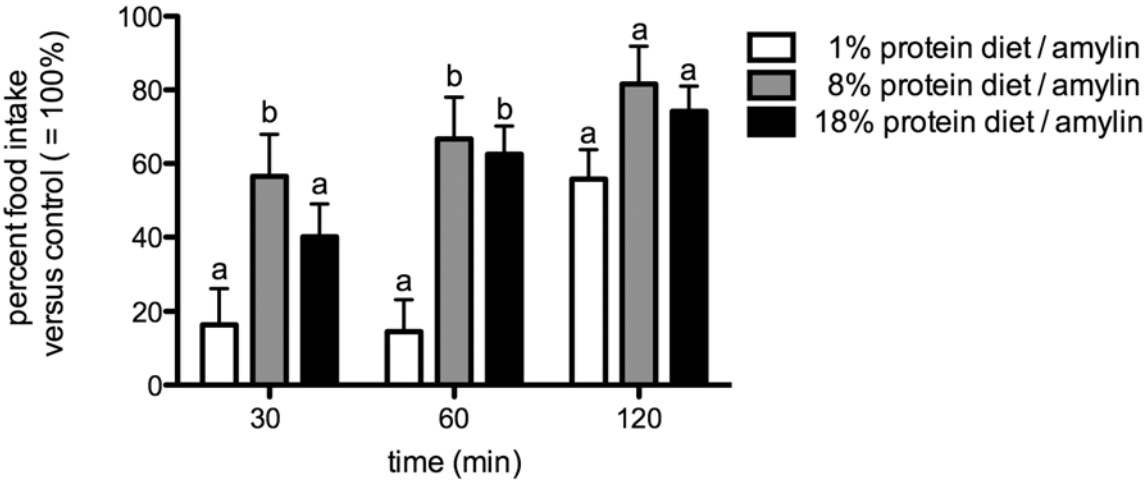


Figure 7 *Food intake of amylin treated rats relative to their respective saline treated controls (=100%).* ***Bars with different letters are significantly different from each other*** *(p < 0.05 one way ANOVA; New Man Keuls post hoc test).*

Effect of an 18% and an 1% protein diet on amylin-induced c-Fos expression in the AP

To investigate the influence of the isocaloric diets on amylin-induced c-Fos expression in the AP, we conducted immunohistological c-Fos studies. We found no difference in the number of c-Fos-IR cells between rats fed 18% fed and 1% at baseline (2.9 ± 1.8 vs. 4.1 ± 1.4 c-Fos-IR cells/section). However, the amylin-induced c-Fos response in the AP of animals that were fed the 18% diet was lower than in animals fed the 1% diet (10.6 ± 3.8 vs. 18.1 ± 2.5 c-Fos-IR cells/section). See **Figure 8**. Representative immunostainings of all experimental groups are shown in **Figure 9**.

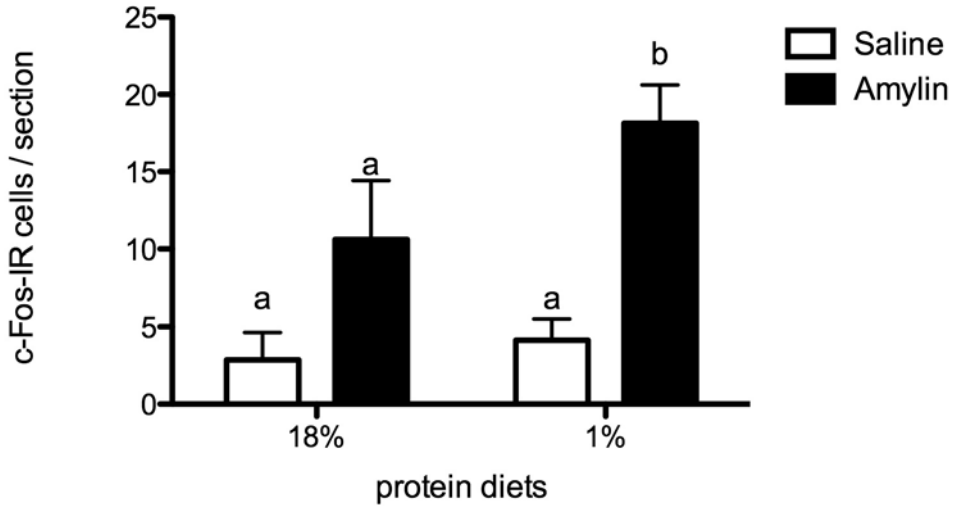
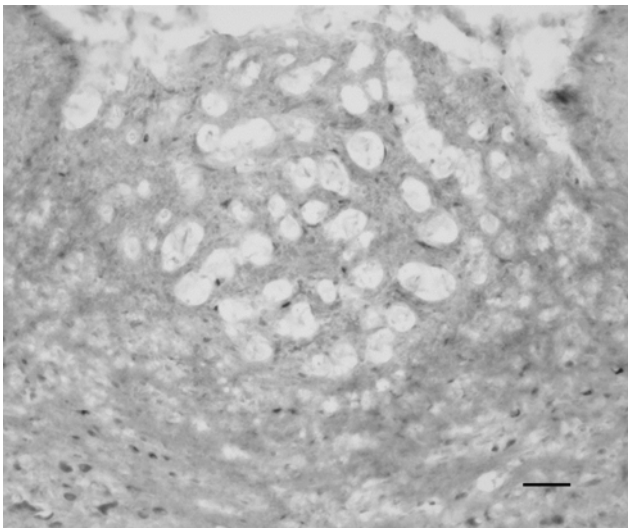
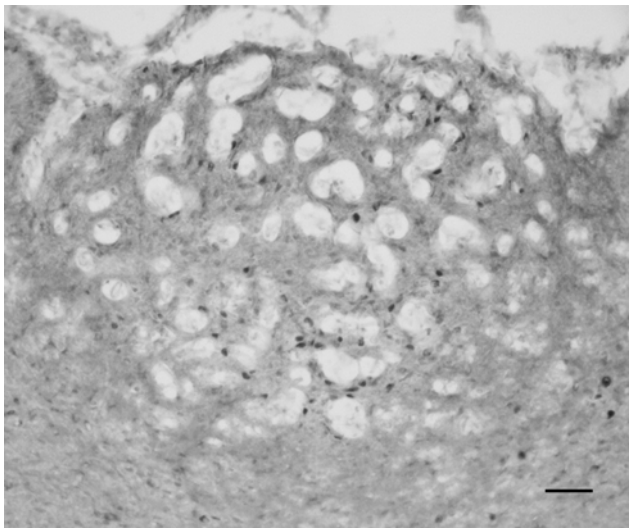


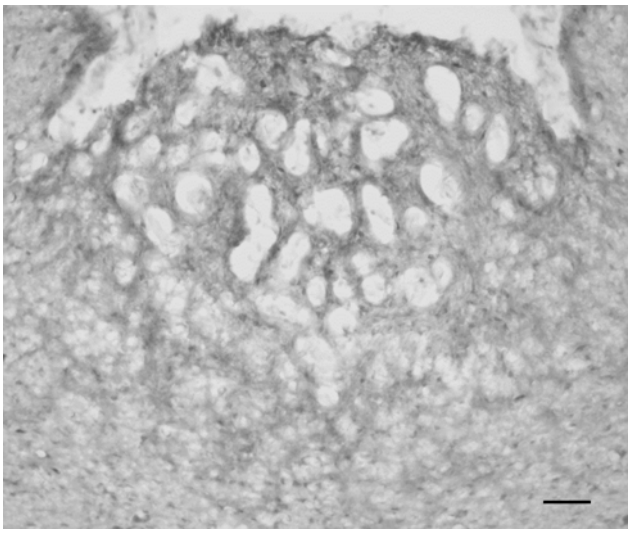
Figure 8 *Quantification of the number of c-Fos-IR nuclei in the AP of ad libitum fed rats that received two different diets (1% or 18% protein) and that were treated with amylin (5µg/kg s.c.) or saline. Data are expressed as means ± SEM (n=7). Bars with different letters are significantly different. (one way ANOVA).*



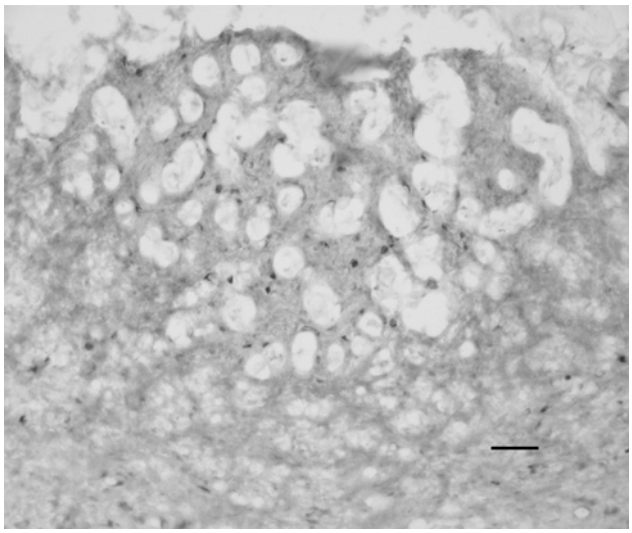
1% protein diet / saline



1% protein diet / amylin



18% protein diet / saline



18% protein diet / amylin

Figure 9 *Representative immunohistochemical c-Fos stainings of 20µm coronal sections of the AP region of rats fed for 48 hrs with the 1% or 18% protein diet and that received amylin (5µg/kg s.c.) or saline; rats were injected at dark onset and perfused two hours later. Left: Under control conditions (1% or 18%/Saline), c-Fos IR cells were almost absent in both diet groups. Right: In rats fed the 1% diet, amylin induced a stronger c-Fos response than in rats fed with the 18% protein diet (scale bar 50µm)*

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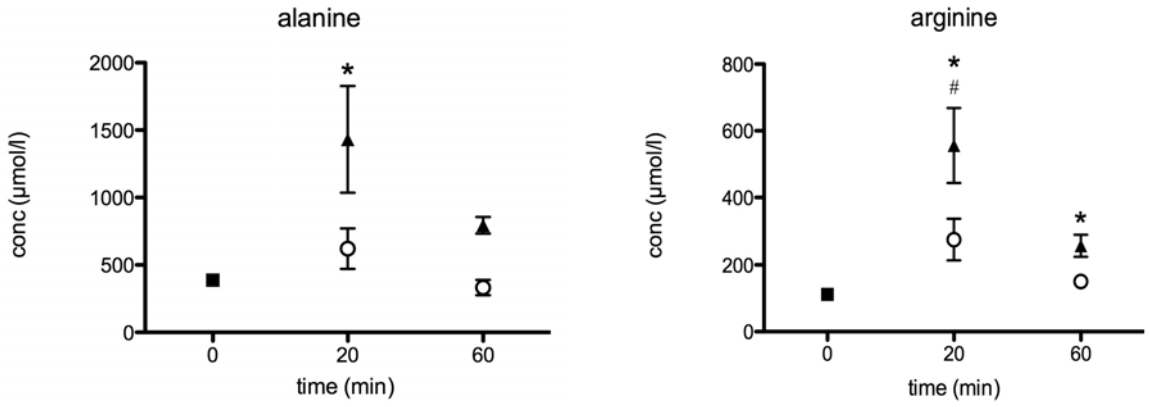
5.2 Effect of peripherally administered amino acids on the anorectic effect of amylin and on the amylin induced c-Fos expression in the AP

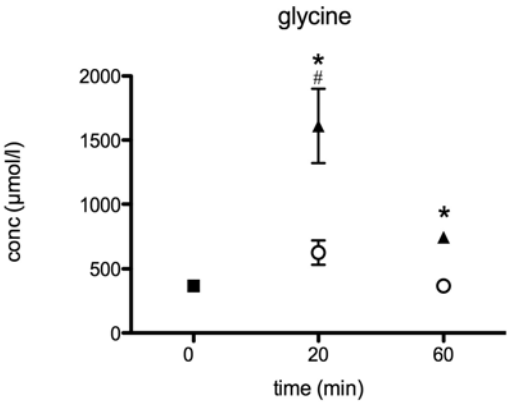
Course of the blood amino acid and hormone concentration after injection of two different doses of amino acids

The effect of Aminoven injection on blood amino acid levels was investigated in 24 hrs fasted rats. The lower Aminoven dose (1g/kg) induced a significant rise of amino acid levels in five of the sixteen amino acids included in the Aminoven solution after 20 min; for most of the other amino acids, there was a trend for an increase after 20 min. All amino acids had nearly returned to baseline by 60 min after injections. The higher Aminoven dose (2g/kg) caused a significant rise of amino acid levels after 20 min in ten out of sixteen amino acids included in the Aminoven solution; most of the amino acids did not return to baseline levels after 60 min and remained elevated. Interestingly, lysine levels actually decreased after 20 min with both Aminoven doses injected. After 60 min, levels had turned back to baseline levels. Overall, the concentration of all amino acids measured showed a (non significant) increase at an Aminoven dose of 1g/kg, the increase was significant for the higher dose injected.

Blood glucose dropped significantly after 20 min in the rats that had received the higher Aminoven dose (2g/kg: 7.7 ± 0.5 to 5.3 ± 0.3 mM $p < 0.05$; 1g/kg: 7.7 ± 0.5 to 6.7 ± 0.5 mM; **Figure 10**).

There was also a significant increase in amylin and leptin levels after 20 min in the 1g/kg treated animals, and in amylin, glucagon, insulin and PP concentrations in the rats injected with 2g/kg Aminoven. In the animals injected with the higher dose, glucagon levels were still increased after 60 min compared to the 20 min value. This was not observed in the rats receiving 1g/kg Aminoven. Further leptin was still significantly increased after 60 min in the 2 g/kg Aminoven injected rats. (**Figure 10**).

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conc (µmol/l)
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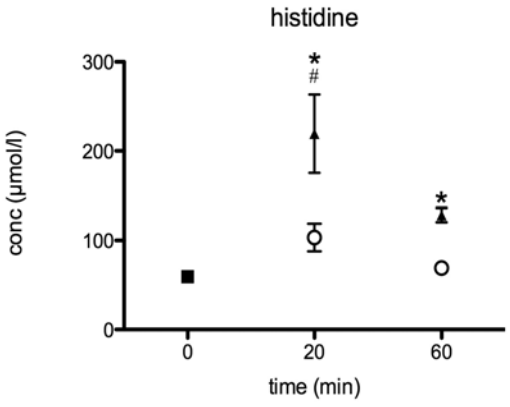
1500
1000
500
0
0
20
60
time (min)
*
#
*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conc (µmol/l)
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0
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60
time (min)
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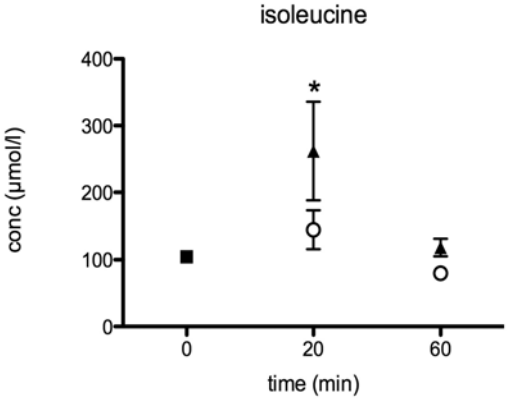
#
*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conc (µmol/l)
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200
100
0
0
20
60
time (min)
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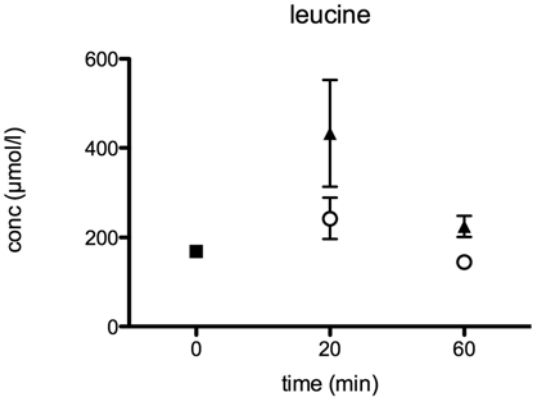
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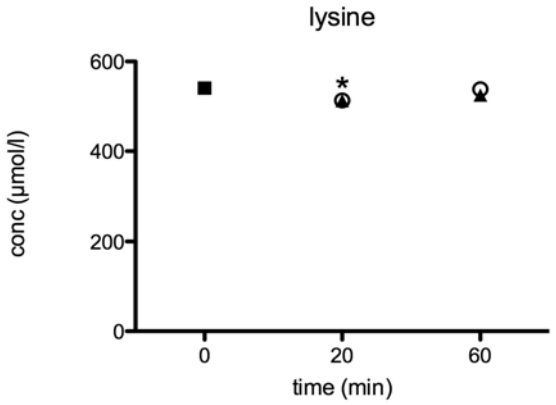
0
20
60
time (min)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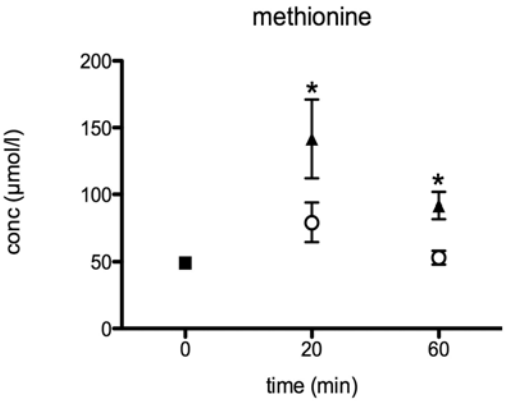
60
time (min)
*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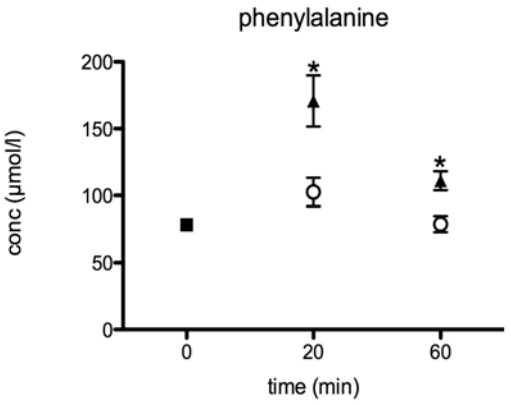
0
0
20
60
time (min)
*
*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0
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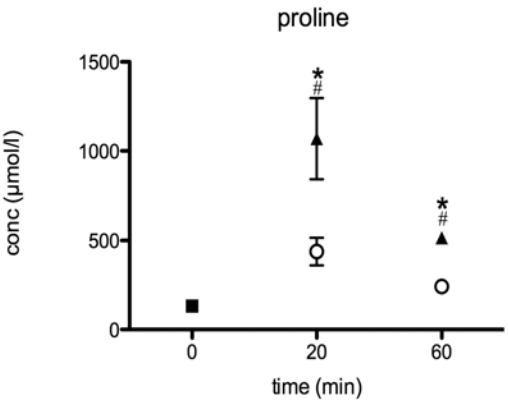
20
60
time (min)
*
*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conc (µmol/l)
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20
60
time (min)
*
#
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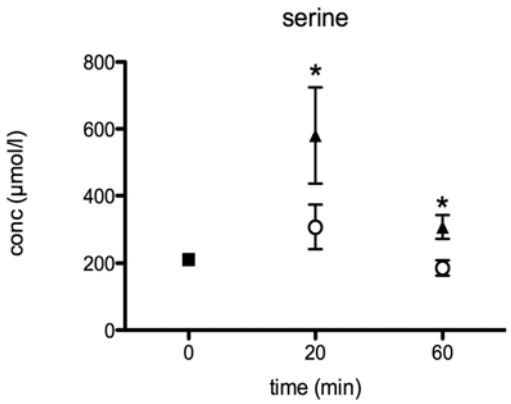
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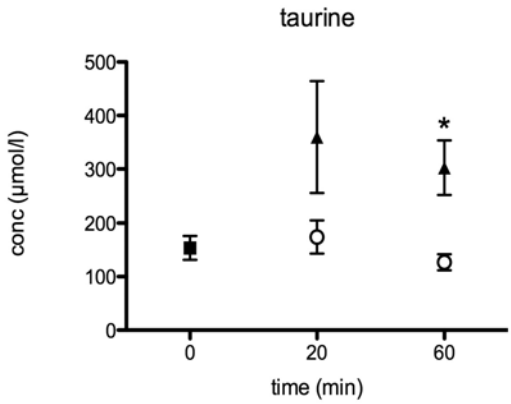
time (min)
*
*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200
100
0
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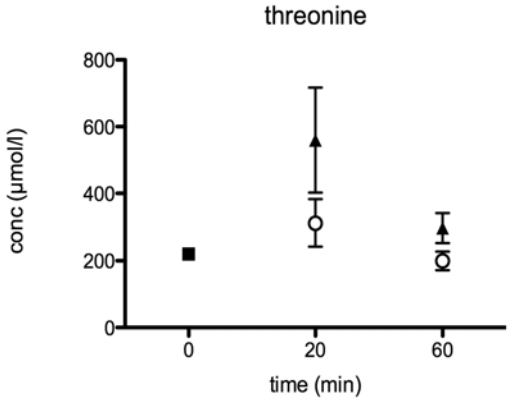
20
60
time (min)
*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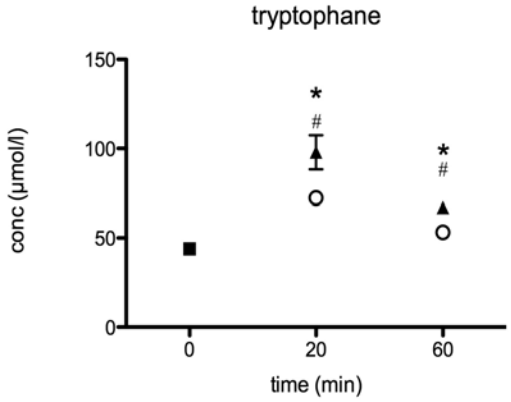
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*
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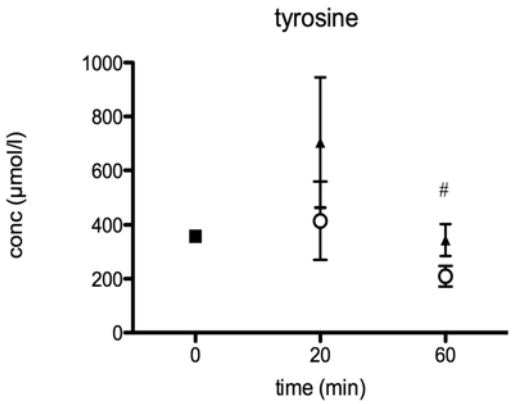
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600
400
200
0
0
20
60
time (min)
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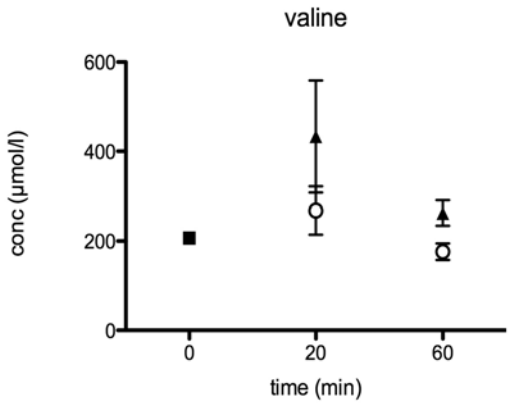
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conc (µmol/l)
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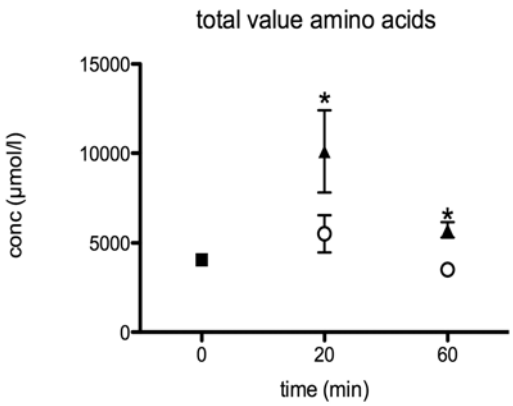
400
200
0
0
20
60
time (min)


total value amino acids
conc (µmol/l)
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10000
5000
0
0
20
60
time (min)
*
*

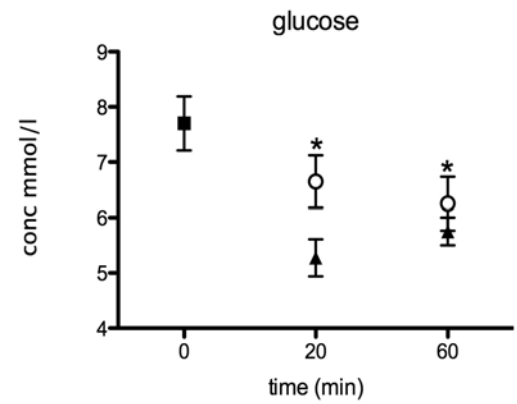

glucose
conc mmol/l
9
8
7
6
5
4
0
20
60
time (min)
*
*

Figure 10 *Blood amino acid levels after injection of two different doses of amino acids: the form of Aminoven ■ = basal value; ○ = 1g/kg ▲ = 2g/kg. Significant differences vs. basal control values: # p < 0.05 (1g/kg Aminoven) ; ★ p < 0.05 (2g/kg Aminoven); unpaired t-test.*

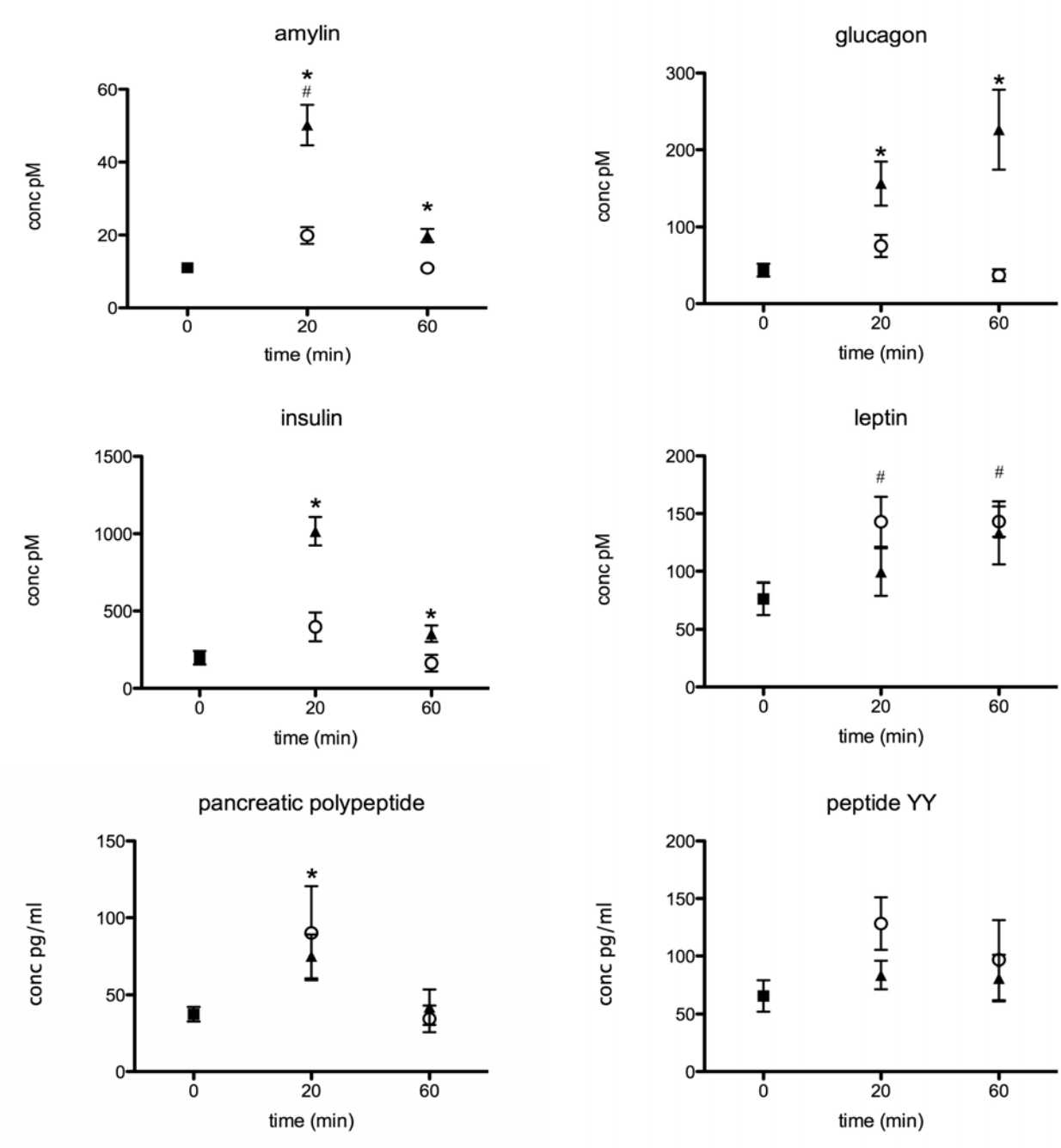


Figure 11 *Blood hormone levels after injection of two different doses of amino acids. ■ = basal value; ○ = 1g/kg amino acids ▲ = 2g/kg amino acids. Significant differences vs. basal control values: # p < 0.05 (1g/kg Aminoven) ; ★ p < 0.05 (2g/kg Aminoven); unpaired t-test.*

Effect of Aminoven on amylin induced c-Fos expression in the AP

Based on the previous experiments, we also tested the influence of an Aminoven injection on the amylin-induced c-Fos expression in the AP. Four experimental groups of 24h fasted rats were treated with saline / saline, saline / amylin, Aminoven / saline or with Aminoven / amylin. Aminoven was given 20 min before amylin which was injected at dark onset.

The quantitative analysis revealed that the amylin-induced c-Fos response in the AP of the saline / amylin treated animals was significantly higher than in the Aminoven / amylin group (43.9 ± 3.5 vs. 25.4 ± 5.1 c-Fos-IR cells/section). Only little c-Fos was found in the saline / saline and Aminoven / saline treated controls. (3.4 ± 2.1 vs. 3.1 ± 1.2 c-Fos-IR cells/section). In the NTS there was no difference between the two amylin treated groups, but the c - Fos expression was significantly higher compared to saline controls. See **Figure 12**, **Figure 13** and **Figure 14** for quantification and for representative immunostainings of all experimental groups.

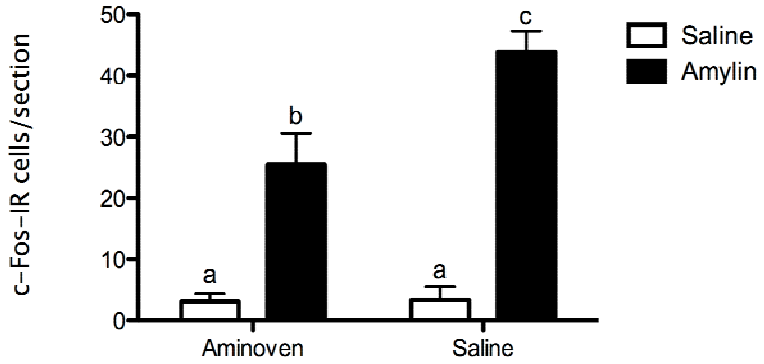


Figure 12 *Effect of Aminoven pre-treatment on the amylin-induced c-Fos expression in the AP of 24h-fasted rats. Aminoven (1g/kg i.p.), amylin (5µg/kg s.c.) (aminoven/saline: n = 8; aminoven/amylin: n = 7, saline/saline: n = 5 , saline/amylin: n = 7). Data are expressed as means ± SEM. Significant differences are indicated by different letters (p< 0.05 one way ANOVA).*

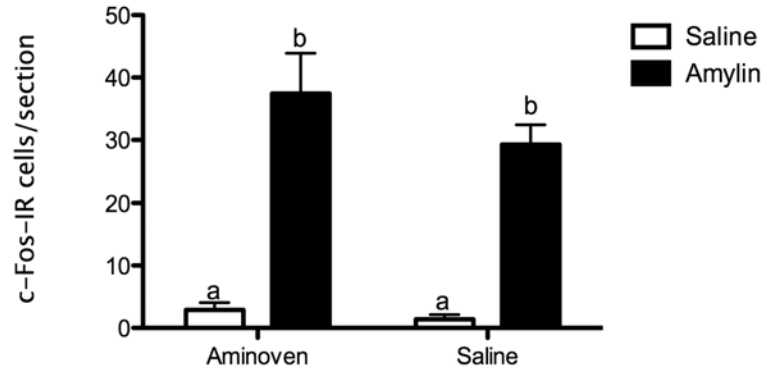
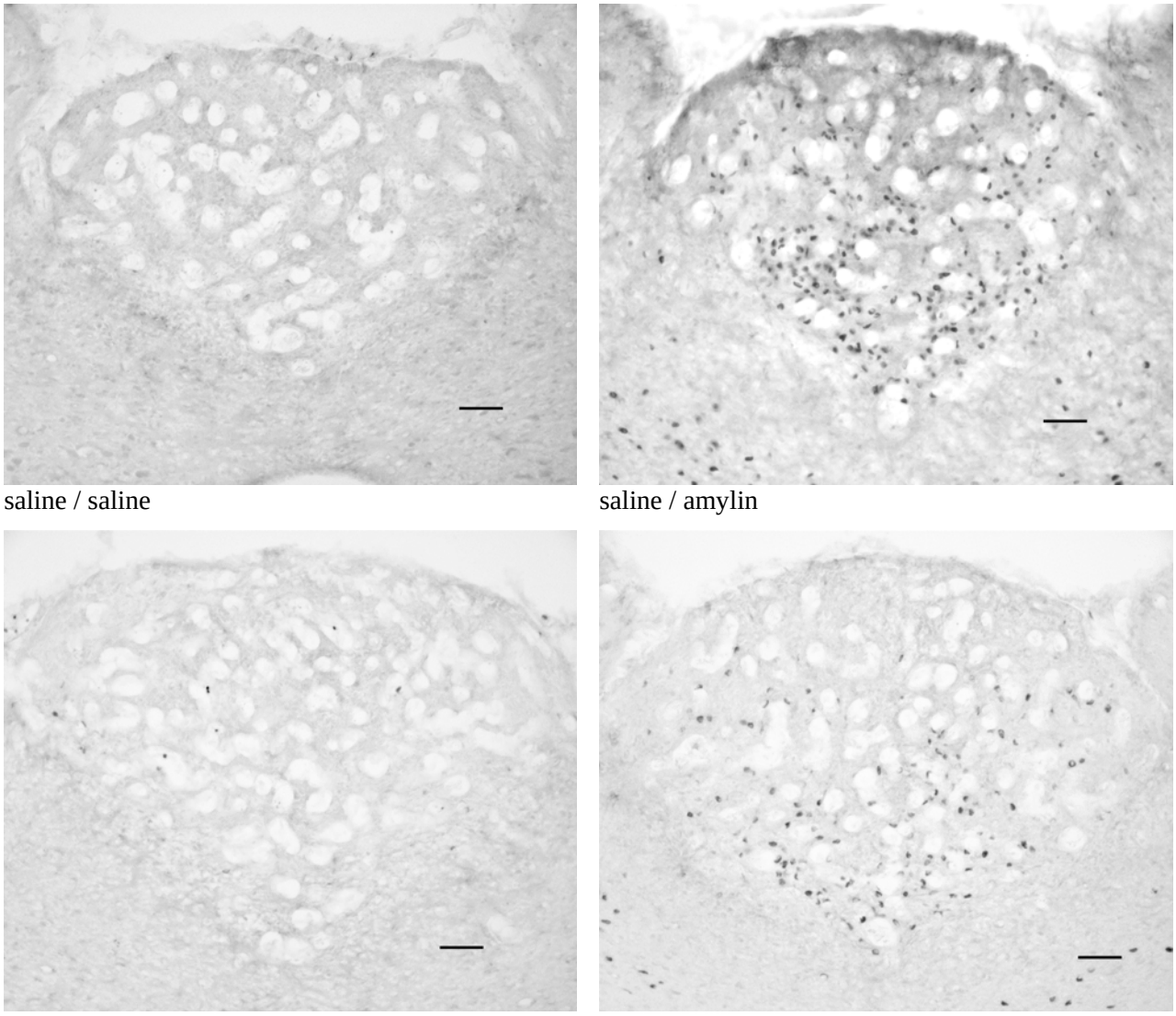


Figure 13 *Effect of Aminoven pre-treatment on the amylin-induced c-Fos expression in the NTS of 24h-fasted rats. Aminoven (1g/kg i.p.), amylin (5µg/kg s.c.) (aminoven/saline: n = 8;*

aminoven/amylin: n = 7, saline/saline: n = 5 , saline/amylin: n = 7). Data are expressed as means ± SEM. Significant differences are indicated by different letters (p< 0.05 one way ANOVA).



saline / saline | saline / amylin

Aminoven / saline | Aminoven / amylin

Figure 14 *Immunohistochemical c-Fos stainings (20µm coronal sections of the AP region) of rats fasted for 24 hrs and treated with Aminoven 1g/kg i.p. or saline and amylin (5µg/kg s.c.) or saline. Left: Under control conditions, c-Fos IR cells were almost absent under both conditions. Right: amylin (5µg/kg) induced a weaker c-Fos response in animals pre-treated with amino acids (scale bar 50µm).*

Influence of Aminoven injection on amylin's inhibitory effect on eating

To test the influence of peripherally injected amino acids on amylin's anorectic effect, the animals were fed with the 1% protein diet. Amylin reduced food intake when rats were fed with this diet for 24 hrs. The effect was significant after 30 min and 60 min compared to controls. Surprisingly prior amino

acid injection (1g/kg) did not alter amylin's inhibitory effect on eating. After 120 min no difference in cumulative food intake was observed among all four groups (**Figure 15***)*

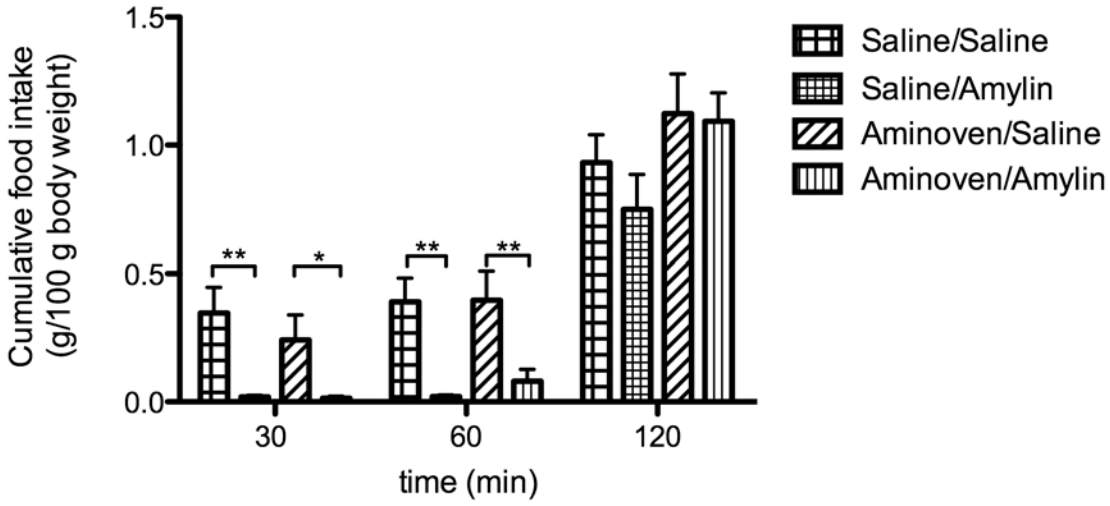


Figure 15 *Effect of amylin (5µg/kg s.c.), injected at dark onset, on food intake in rats kept on the 1% protein diet for 24 hrs. Rats were injected with Aminoven (1g/kg i.p.) 20 min before dark onset. Bars represent group means ± SEM. (n = 12) * p <0.05, *p <0.01 and ***p <0.001 significantly different from respective control (saline) group (paired Student t test within saline or aminoven groups, respectively).*

5.3 Effect of glucagon on the amylin induced c-Fos expression in the AP

Influence of glucagon on the c-Fos expression induced by amylin

In this experiment, the animals were distributed into six experimental groups to test if glucagon has an acute effect on the amylin induced c-Fos expression in the AP.

At both doses tested, glucagon alone did not induce a c-Fos response in the AP and NTS. Furthermore, there was no significant difference in the number of c-Fos positive cells after amylin among the groups irrespective of whether they had been treated with glucagon or not (**Figure 16** and **Figure 17**). Representative immunostainings of all experimental groups are shown in **Figure 18.**

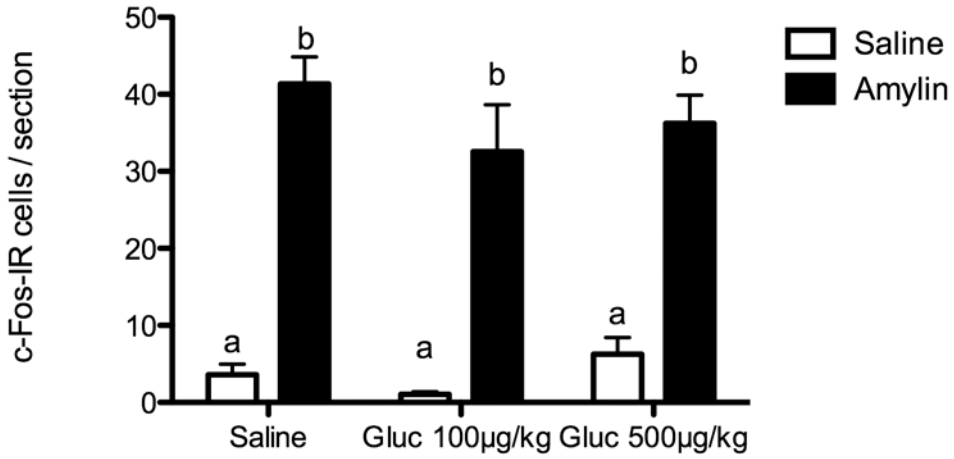


Figure 16 *Quantification of the number of c-Fos-IR nuclei in the AP in 24 hrs fasted rats that received either saline and glucagon (100µg/kg or 500µg/kg i.p) and saline or amylin, respectively (saline/saline: n = 6; saline/amylin: n = 8, glucagon 100µg/kg/saline: n = 5 , glucagon 100µg/kg /amylin: n = 8, glucagon 500µg/kg/saline: n = 6 , glucagon 500µg/kg/amylin: n = 6). Data are expressed as means ± SEM. Bars with different letters are significantly different (one way ANOVA).*

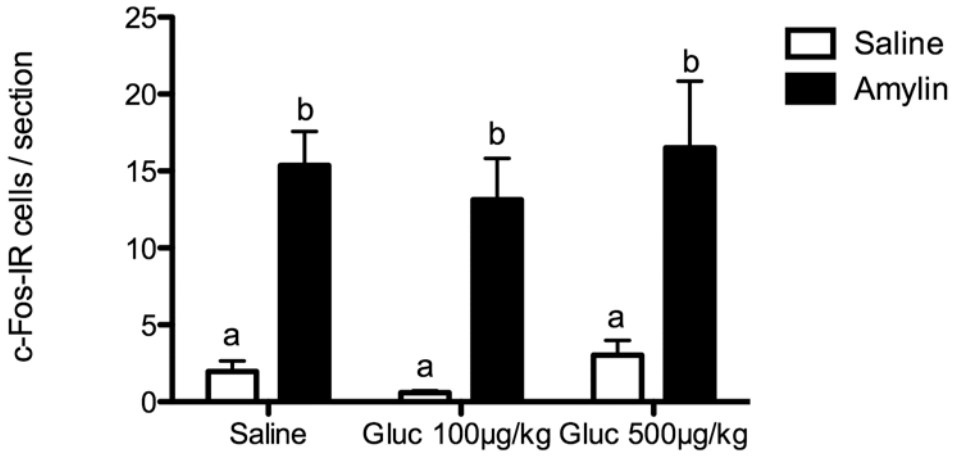
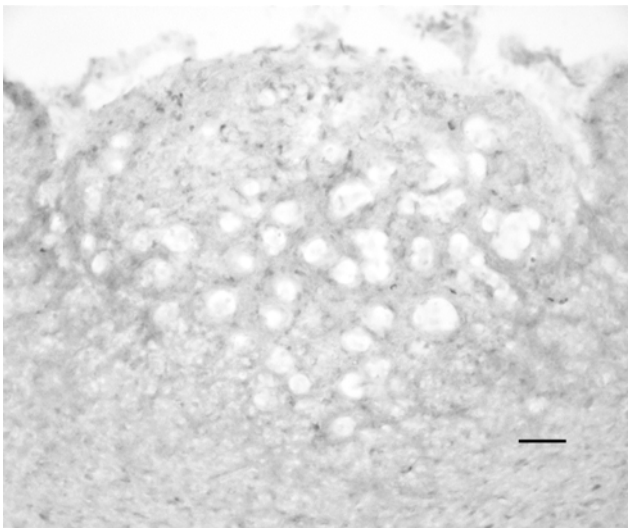
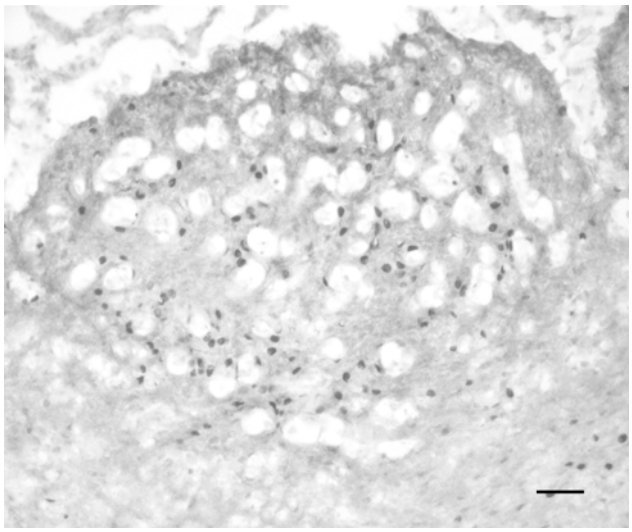


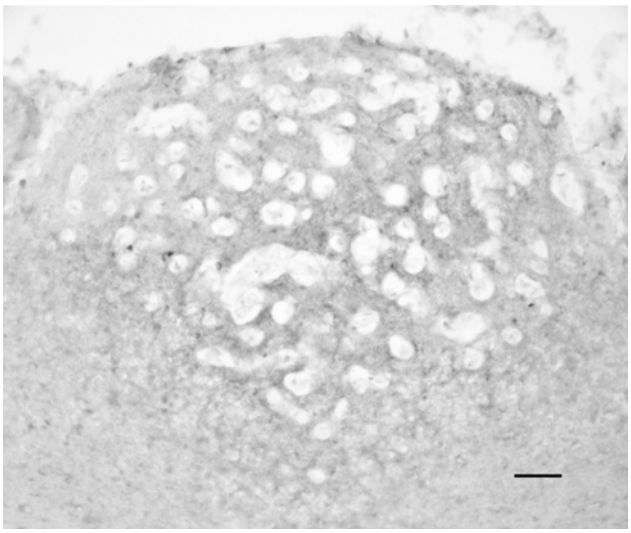
Figure 17 *Quantification of the number of c-Fos-IR nuclei in the NTS in 24 hrs fasted rats that received either saline and glucagon (100µg/kg or 500µg/kg i.p) and saline or amylin, respectively (saline/saline: n = 6; saline/amylin: n = 8, glucagon 100µg/kg/saline: n = 5 , glucagon 100µg/kg /amylin: n = 8, glucagon 500µg/kg/saline: n = 6 , glucagon 500µg/kg/amylin: n = 6). Data are expressed as means ± SEM. Bars with different letters are significantly different (one way ANOVA).*



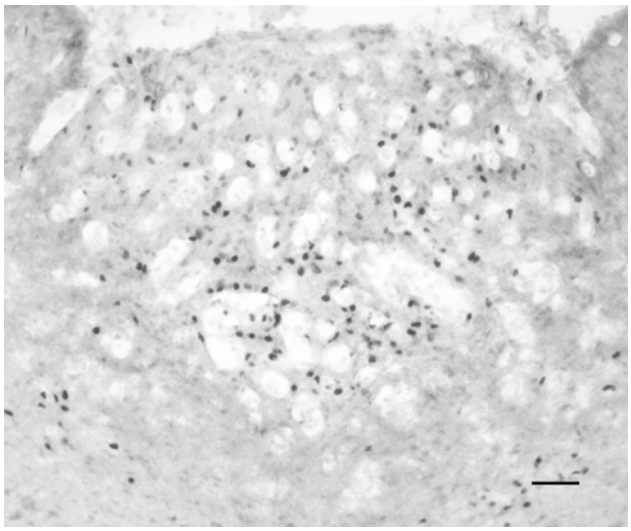
saline / saline



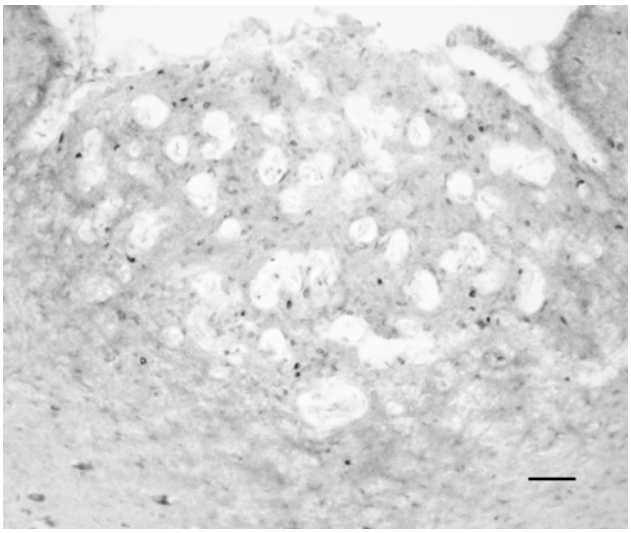
saline / amylin



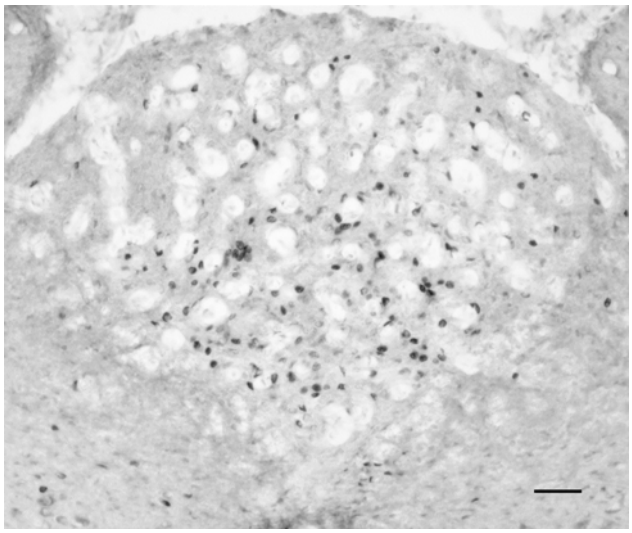
glucagon 100µg/kg / saline



glucagon 100µg/kg / amylin



glucagon 500 µg/kg / saline



glucagon 500µg/kg / amylin

Figure 18 *Representative immunohistochemical c-Fos stainings of 20µm coronal sections of the AP region of rats fasted for 24 hrs and treated with saline or glucagon (100µg/kg or 500µg/kg i.p.) or saline and amylin (5µg/kg s.c.), respectively . Left: Under control conditions (saline/glucagon*

100µg/kg/glucagon 500µg/kg) c-Fos IR cells were almost absent. Right: All three groups treated with amylin (5µg/kg) had a similarly strong c-Fos response irrespective to their glucagon treatment (scale bar 50µm).

6 Discussion

The aim of the present study was to extend the findings of Michel (Michel et al., 2007) that diet-derived protein attenuates the effects of amylin on c-Fos expression in the AP and on food intake. In contrast to these previous studies using non-caloric mash that was selectively supplemented with nutrients, isocaloric diets with different protein content were used in the present study. This largely eliminated the possibility that differences in caloric intake exerted a confounding influence on the experimental outcome. The same holds true for possible differences in blood glucose levels, which did not differ between animals fed the different protein diets in the current experiments.

The mechanisms underlying the protein-dependent suppression of amylin responsiveness as determined by c-Fos immunocytochemistry and amylin's effect on eating are still unknown. Because a given dose of exogenous amylin resulted in similar plasma amylin concentrations irrespective of the protein content of the diet, modulation of amylin responsiveness is most likely due to a post - receptor effect and not due to a difference in the metabolism (and hence circulating half life of exogenous amylin). To test whether a postabsorptive increase in circulating amino acids might be a contributing factor, the effect of an intraperitoneal amino acid injection on amylin-induced anorexia and AP activation was determined in rats fed the 1 % protein diet. Amino acid injection lead to an acute elevation of circulating amino acids, without prior passage and absorption via the gastrointestinal tract. It is also an open question whether hormonal stimuli might be indirectly involved in the protein de-pendent attenuation of amylin responsiveness. At least with respect to the control of glucose homeostasis, glucagon is a functional counterplayer to amylin (and insulin). Moreover, blood glucagon levels are known to increase in particular in response to protein intake (Geary et al., 1981). However, neurophysiological studies characterizing the effect of glucagon in the central nervous system are scarce. Therefore we also tested the possible interaction between exogenous glucagon and amylin in c-Fos studies.

6.1 Effect of isocaloric diets with different protein contents on the anorectic effect of amylin and on the amylin-induced c-Fos expression in the AP/NTS

The acceptance of the different diets was similar. There was no difference in total food intake of the 1% protein diet vs. the 18% protein diet. Hence, the lower total blood amino acid concentrations of the 1% protein diet vs. the 18% protein diet are due to the different protein content of the diets. The lower blood amino acid concentration under the 8% protein diet vs. the 18% protein also appears to be a consequence of the different protein contents of these diets. It cannot be excluded, however, that slightly lower baseline intake of the 8% protein diet might have contributed to the decreased concentration of blood amino acids. The reason for the lower intake of the 8% protein diet remains unknown at present.

Since all diets were isocaloric, it is unlikely that differences in energy intake account for the differences in amylin responsiveness under the different feeding conditions. The current studies also most likely rule out that diet-dependent differences in blood glucose might have had an influence on the effects of amylin. This issue is of particular interest because amylin-sensitive neurons have been shown to be glucose-responsive (Adachi et al., 1995; Riediger et al., 2002). Furthermore, amylin's inhibition of gastric emptying which also depends on the AP is overridden by hypoglycemia (Young et al., 1999); the latter has been interpreted to be based on the co-sensitivity of AP neurons to amylin and glucose. Previous studies suggested that a low glucose concentration of (approximately 3.5 mM) in rats fed a lard containing non-caloric mash did not prevent amylin-induced anorexia and c-Fos expression in the AP (Michel et al., 2007). Therefore a glucose-dependent attenuation of amylin's action on AP neurons does not seem to occur unless hypoglycemia is more pronounced. Hence, such effects cannot account for the differences in amylin signaling under our experimental, euglycemic conditions, particularly because there were no differences in glucose levels among the diet groups.

Our studies demonstrate that amylin's anorectic action is stronger in rats fed a 1% protein diet compared to the 8% or 18% protein diets, indicating that diet-derived protein attenuates amylin's suppressive effect on food intake. This result is consistent with and extends previous experiments (Michel et al., 2007) investigating the effect of diet-derived nutrients on amylin-responsiveness using test diets that contained only one single macronutrient (glucose, lard, protein). According to the different protein contents, the diets produced significantly different levels of blood amino acids (highest value for 18% diet). Hence, a postabsortive effect of amino acids is a possible factor that might be relevant for the modulation of amylin-sensitivity under the different diet conditions that we tested here.

Interestingly, amylin's anorectic effect was similar in rats receiving the 8% and 18% protein diets. Therefore, dynamic effects of the protein content on amylin responsiveness seem to occur at protein contents of the diet lower than 8%, at least when an animal is fed ad libitum. Notably, under free living conditions, protein supply is likely to vary, both with respect to the frequency of intake (food availability) and in terms of the diet's protein content. For these reasons short-term excursions in protein supply might modulate amylin-responsiveness under normal feeding conditions. This is supported by recent unpublished follow-up studies showing that a 5h period of food deprivation is sufficient to increase amylin-induced activation of AP neurons (c-Fos) relative to ad libitum chow-fed controls (unpublished results).

To test whether the attenuation of amylin's anorectic action is paralleled by a reduced amylin-mediated AP activation, the effects of amylin on c-Fos expression were compared in rats fed with a 1% vs. an 18% protein diet. In line with the observations of the behavioral experiments, amylin induced a lower c-Fos response in animals receiving the 18% protein diet. The excitatory action of amylin on AP neurons is considered the primary neuromechanism underlying amylin's feeding

inhibitory effect. It is therefore conceivable that the impact of diet-derived protein on amylin's feeding effect is mediated by an attenuation of amylin-responsiveness of AP neurons.

It might appear unexpected that despite a low c-Fos response, amylin produced a significant anorectic response in rats receiving the 18% protein diet. However, these results are consistent with our previous studies (Michel et al., 2007) conducted with nutritionally equivalent standard chow. Both the amylin-induced c-Fos expression and the anorectic response to amylin were similar to the current results. In general, the following consideration has to be taken into account. Although c-Fos is an accepted and useful marker for neuronal activation, the thresholds for amylin-induced c-Fos expression, amylin-induced neuronal activation and amylin's effects on feeding do not necessarily correspond. The current studies are not designed to correlate these parameters directly, but they support the concept that a protein-dependent decrease in neuronal amylin responsiveness (c-Fos expression) is associated with a decreased anorectic effect of exogenous amylin. This has to be clearly segregated from the question whether the thresholds for these effects are the same.

In summary the current studies confirmed that blood amino acid concentrations change depending on the protein content of the diet, and that amylin`s anorectic action is stronger in rats fed a 1% protein diet compared to 8% or 18% protein diets. Amylin responsiveness of AP neurons is attenuated by diet-derived protein. This effect does not depend on differences in caloric intake or blood glucose levels.

6.2 Effect of peripherally administered amino acids on the anorectic effect of amylin and on the amylin induced c-Fos expression in the AP

Based on the assumption that diet derived protein attenuates amylin responsiveness, we investigated if peripherally injected amino acids attenuate amylin's anorectic effect and the amylin induced c-Fos response in the AP/NTS.

Both doses of Aminoven (1g/kg or 2g/kg) produced transient increases in blood amino acid levels, although this increase was not always significant for all of the in Aminoven contained amino acids. Blood glucose and hormone (e.g. insulin) levels are known to change in response to an increase in blood amino acid levels; it was e.g. shown by Liu et al. (2008) that l-arginine, l-lysine, l-alanine, l-proline, l-leucine and l-glutamine acutely stimulate insulin secretion from mouse islets in a dose-dependent manner. Under our conditions blood glucose values decreased transiently only in response to the higher amino acid dose. Similarly, the higher dose of amino acids resulted in a stronger stimulation of pancreatic (e.g. insulin, glucagon) and gastrointestinal hormone release (e.g. PYY). To limit the possible impact of these factors, the lower dose of Aminoven was chosen for the subsequent c-Fos and feeding experiments.

Our studies clearly show that a dose of amylin (5µg/kg s.c.) that induces a strong c-Fos response in 24 h fasted rats (Michel et al., 2007) is attenuated by peripherally injected amino acids (1g/kg). In the control group receiving amino acids without amylin, only a low number of c-Fos positive cells was present in the AP. This indicates that the amino acid stimulus per se did not affect c-Fos expression.

The neuronal activation pattern in the NTS was similar to the effects observed in the AP. This is in line with previous demonstrations that the amylin induced c-Fos expression in the NTS is secondary to the activation of AP neurons (Riediger et al., 2004).

The amino acid dependent attenuation of the amylin-induced AP activation did not translate into an attenuation of amylin's hypophagic feeding response in animals fed a 1% protein diet. In other words amylin reduced eating in rats fed this diet irrespective of whether they had received a prior Aminoven injection or not. Whether the effect of Aminoven on amylin-induced AP activation was not strong enough to affect amylin's feeding response or whether such an effect might have been masked by counteracting mechanisms is unclear. Such mechanisms might involve the effect of anorectic hormones (e.g. glucagon) stimulated by the amino acid treatment. Although we tried to keep the effect of the Aminoven treatment on hormone secretion (e.g. insulin, glucagon, PP, PYY, leptin) small, we cannot exclude the possibility that hormonal stimuli interfered with the effect of amino acids (see also 6.3). It appears unlikely, however that the Aminoven-stimulated amylin secretion that we observed might have counteracted a possible attenuation of amylin responsiveness by the amino acid treatment. In relation to the increase in plasma amylin levels produced by the amylin injection in rats fed with the different protein diets for 24 hrs, the increase in endogenous amylin induced by Aminoven treatment appears to be too low. Overall and based on our findings we conclude that an acute increase in blood amino acid levels attenuates the amylin induced c-Fos response. Under the current experimental condition we could not demonstrate an attenuation of amylin's anorectic effect by peripherally applied amino acids.

6.3 Effect of glucagon on the amylin induced c-Fos expression in the AP

To further investigate possible indirect mechanisms involved in the protein-dependent reduction of the amylin induced AP activation we tested if the pancreatic hormone glucagon might play a role. Plasma glucagon levels increase during protein meals in rats and humans (Geary et al., 1981), and amino acids (especially arginine, alanine and glutamine) are potent stimulators of glucagon secretion (Pipeleers et al., 1985; Young and Denaro, 1998; Dumonteil et al., 2000).

Based on the documented anorectic action of glucagon (e.g. Geary and Smith, 1982; Le Sauter et al., 1991; Geary et al., 1993; Lutz et al., 1996) it might appear counterintuitive to assume that glucagon release might be involved in the reduction of amylin responsiveness mediated by diet-derived protein. However, despite the accepted anorectic effect of glucagon, some studies demonstrate a glucagon-mediated increase in food intake under some experimental conditions (Hell and Timo-Iaria, 1985). Furthermore, glucagon is a physiological counterplayer to amylin and insulin action, at least with respect to the control of glucose homeostasis (Young et al., 1993; Lutz et al., 1998b; Lutz et al., 2001; Young, 2005b).

In our hands, however, acute injections of two different glucagon doses did not attenuate the amylin-induced c-Fos response in the AP, which at least argues against a short-term interaction between

amylin and glucagon with respect to neuronal AP activity. Further, at both doses, glucagon alone did not elicit an appreciable c-Fos expression in the AP. It remains to be clarified whether an elevation of blood glucagon levels over a longer period of time than under the acute setting might have an impact on amylin signalling.

6.4 Perspectives and significance

The current studies further support and extend the evidence that diet-derived nutrients, in particular protein, attenuate amylin's AP-mediated suppression of food intake; however, the cellular mechanisms still need to be identified. In relation to this question, it is of interest whether the protein-dependent attenuation of neuronal AP sensitivity is specific to amylin or whether other hormonal stimuli are also affected. Amylin sensitive neurons have been shown to be co-sensitive to the anorectic hormone glucagon-like peptide 1 (Riediger, 2000). Hence, it would be interesting to test whether fasting also increases the GLP-1 mediated activation (c-Fos) of AP neurons and whether this effect is protein dependent. A negative outcome would suggest a specific effect of diet-derived protein on amylin responsiveness. A positive outcome might suggest that the protein-mediated attenuation of neuronal sensitivity may be specific for amylin-sensitive neurons but not restricted to a single excitatory stimulus. In the latter case, it might be interesting to test the effect of fasting on other hormonal stimuli that are unrelated to the control of food intake. Such a stimulus could be angiotensin II, which modulates baroreceptor function via the AP (McKinley et al., 1992). Preliminary results conducted in a follow-up study seem to indicate that the angiotensin II induced c-Fos expression in the AP is not enhanced by fasting. Moreover, angiotensin II sensitive neurons do not seem to express the CTR, indicating that these cells are not amylin sensitive because the CTR represents the necessary core component of functional amylin receptors. This suggests indirectly that the increase in sensitivity of AP neurons induced by fasting and by a decreased protein intake in the diet might be specific for amylin responsive cells.

The current findings imply that there is an inverse relation between amylin responsiveness and protein intake. Although this effect is unrelated to caloric intake per se it affects the amylin-induced inhibition of food intake. One possible physiological implication might be that intake of metabolically more important fuels in omnivores (carbohydrates and fats) is less strongly inhibited when their content in the diet is low relative to protein. A further possible implication might be that other AP-dependent actions of amylin are also modulated by the protein intake. At least the inhibitory effect of amylin on gastric emptying is mediated by the AP (Young, 2005b). Based on the current findings one may speculate that the amylin-dependent inhibition of gastric emptying is stronger after a period of fasting or low protein intake. Such a mechanism might adapt digestive function to a sudden increase in food or protein intake. It was beyond the scope of the current studies to investigate these issues.

In addition to possible physiological implications, there might be a therapeutic relevance because amylin analogs are promising drugs for the treatment of obesity and associated metabolic disorders (diabetes mellitus). Based on our current findings the effectiveness of amylin analogs could be modulated by the protein intake, which might affect the treatment outcome.

7 Abbreviations

A	adrenergic neurons
AM	adrenomedullin
AP	area postrema
BBB	blood brain barrier
BSTL	bed nucleus of the stria terminalis
CCK	cholecystokinine
Ce	central nucleus of the amygdala
CGRP	calcitonin - gene related peptide
CNS	central nervous system
CTR	calcitonin receptor
CVO	circumventricluar organ
GLP1	glucagon like peptide 1
lPB	lateral parabrachial nucleus
NA	noradrenergic neurons
NCM	non caloric mash
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
PB	phosphate buffer
POMC	proopiomelanocortin
PP	pancreatic polypeptide
PYY	peptide tyrosine tyrosine
RAMP	receptor activity modifying proteins
SFO	subfornical organ
SSA	sulfosalicyclic acid solution
UPLC	Ultra Performance Liquid Chromatography

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10 Curriculum vitae

Name	Karoline Forster
Date of birth	09. December 1981
Place of birth	Vienna, Austria
Nationality	Austrian
Mother	Ing. Margit Forster, name at birth Lippitsch
Father	Dr. med. vet. Herfried Forster, Veterinarian in Graz

1992 – 2000	High school in Graz, Austria
2000	High school degree, high school Lichtenfelsgasse, Graz Matura

2000 – 2006	Study of veterinary medicine at the University of Veterinary Medicine in Vienna, Austria
01/2006	Graduations at the University of Veterinary Medicine in Vienna, Austria

2006 – 2007	employed as assistant in the clinical practice of my father in Graz, Austria
2007 – 2008	Internship for small animals at the Justus Liebig University, Giessen, Germany
2008 – 2011	Dissertation at the Institute of Veterinary Physiology at the Vetsuisse Faculty University of Zürich, Switzerland
since 01/2010	Assistant in the Clinic for Small Animal Medicine at the Vetsuisse Faculty University of Zürich, Switzerland

Zürich, February 2011